SEQUENCES CHARACTERISTIC OF HYPOXIA-REGULATED GENE TRANSCRIPTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part application of U.S. application no. 09/383,096, filed August 27, 1999, which is a continuation-in-part of U.S. application no. 09/138,109, filed August 21, 1998, and a conversion of U.S. provisional applications no. 60/098,158, filed August 27, 1998 and no. 60/132,684, filed May 5, 1999, the entire contents of all of which are hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

Technical Field

[0002] The present invention relates to the identification of genes that are differentially expressed in hypoxia and use of the genes and gene products for diagnosis and therapeutic intervention. The invention further relates to identification of polynucleotide sequences, and their gene products, that are differentially expressed in hypoxia and the use of the sequences for diagnosis and probes.

Background Art

[0003] The level of tissue oxygenation plays an important role in normal development as well as in pathologic processes

such as ischemia. Tissue oxygenation plays a significant regulatory/inducer role in both apoptosis and in angiogenesis (Bouck et al, 1996; Bunn et al, 1996; Dor et al, 1997; Carmeliet et al, 1998). Apoptosis (see Duke et al, 1996 for review) and growth arrest occur when cell growth and viability are reduced due to oxygen deprivation (hypoxia). Angiogenesis (i.e. blood vessel growth, vascularization) is stimulated when hypo-oxygenated cells secrete factors which stimulate proliferation and migration of endothelial cells in an attempt to restore oxygen homeostasis (for review see Hanahan et al, 1996).

[0004] Hypoxia plays a critical role in the selection of mutations that contribute to more severe tumorogenic phenotypes (Graeber et al, 1996). Identifying activated or inactivated genes and gene products in hypoxia and ischemia is needed.

[0005] Ischemic disease pathologies involve a decrease in the blood supply to a bodily organ, tissue or body part generally caused by constriction or obstruction of the blood vessels, as for example retinopathy, myocardial infarction and stroke. Therefore, apoptosis and/or angiogenesis as induced by the ischemic condition are also involved in these disease states. Neoangiogenesis is seen in some forms of retinopathy and in tumor growth. These processes are complex cascades of

events controlled by many different genes reacting to the various stresses such as hypoxia.

[0006] Stroke is the third leading cause of death and disability in developed countries, affecting more than half a million Americans each year. Stroke is an acute neurologic injury occurring as a result of an insult to the brain, thus interrupting its blood supply. Stroke induces neuronal cell death, which leads to the clinical outcomes of patients' death or disability ranging from total paralysis to milder dysfunction. Cerebral ischemia is the most common type of stroke, which may lead to irreversible neuronal damage at the core of the ischemic focus, whereas neuronal dysfunction in the penumbra may be reversible. Cells in the penumbra have an estimated time window for survival of up to 6 hours. The ability to intervene as soon as the patient is identified is essential for recovery. It is well established that ischemic tissue damage is multifactorial and involves at least excitotoxicity, reactive oxygen species, and inflammation - all leading to neuronal cell death.

[0007] Treatment strategies for stroke are aimed to induce rapid reperfusion and rescue of neurons in the penumbral area. Neuroprotective drugs are constantly being developed in an effort to rescue neurons in the penumbra from dying. However, potential cerebroprotective agents need to counteract all the

above-mentioned destructive mechanisms. Therefore, current therapy in stroke focuses primarily on prevention, minimizing subsequent worsening of the infarction, and decreasing edema.

[0008] The ability to monitor hypoxia-triggered activation of genes can provide a tool to identify not immediately evident ischemia in a patient. Identification of hypoxia-regulated genes permits the utilization of gene therapy or direct use of gene products, or alternatively inactivation of target genes for therapeutic intervention in treating the diseases and pathologies associated with hypoxia, ischemia and tumor growth.

[0009] Induction of p53 in response to hypoxia and DNA damage and its ability to inhibit cell growth in response to common cellular stresses, is a major function associated with its role as a tumor suppressor gene (Lane, 1992). Proteins encoded by p53 target genes have been shown to regulate various processes controlling growth and viability of tumor cells, such as cell cycle progression and programmed cell death. Like p53, the growth arrest and DNA damage (GADD) genes are induced in cells exposed to genotoxic stress. GADD genes were originally identified by subtraction hybridization from a cDNA library constructed from UV-irradiated Chinese hamster ovary cells (Forance et al, 1989). The GADD genes code for a diverse range of proteins with a variety of

functions, including the suppression of DNA synthesis (Smith et al, 1994), the inhibition of differentiation (Batchvarova et al, 1995) and the induction of apoptosis (Takekawa et al, 1998). The response to genotoxic stress of some GADD genes is rapid but transient whereas others respond more slowly (Fleming et al, 1998). Other stimuli, such as DNA damage or contact inhibition, also increase gene expression. The regulation of these genes by stress is complex and appears to be mediated by multiple pathways. For example, ionizing radiation induces the transcription of GADD45, which inhibits proliferation and stimulates DNA excision repair, through a p53-dependent mechanism (Hollander et al, 1993). In contrast, UV irradiation increases GADD45 expression in the absence of p53 binding directly to the GADD45 promoter (Zhan et al, 1996). GADD45 mRNA levels are also increased during hypoxia, focal cerebral ischemia, and after exposure of cells to agents which elevate the levels of the glucose-regulated proteins (Price et al, 1992, Schmidt-Kastner et al, 1998). In addition to its ability to inhibit proliferation and stimulate DNA repair, GADD45 can also induce apoptosis when overexpressed in cells *in vitro* (Takekawa et al, 1998).

[0010] Recently, a novel p53 target gene and member of the GADD family, PA26 was identified (Velasco-Miguel et al, 1999). PA26 encodes at least three transcript isoforms, of which two

are differentially induced by genotoxic stress in a p53-dependent manner. The function of PA26 is unclear.

SUMMARY OF THE INVENTION

[0011] The present invention provides purified, isolated and cloned polynucleotides (nucleic acid sequences) associated with hypoxia-regulated activity and having sequences designated as any one of SEQ ID NOs:1-12, or having complementary or allelic variation sequences thereto. The expression of these polynucleotides is modulated when cells are subjected to neurotoxic stress. The present invention includes the polynucleotides of SEQ ID NOs:1-12, as well as the naturally-occurring full-length RNAs and corresponding full-length cDNAs which include any one of these sequences.

[0012] The invention is further directed to naturally-occurring polynucleotides having at least 70% identity with any of the polynucleotides which include any one of SEQ ID NOs:1-12, or which are capable of hybridizing under moderately stringent conditions to any of such polynucleotides, and whose expression in naturally-occurring neural cells is modulated when the cells are subjected to hypoxic stress.

[0013] The present invention is also directed to fragments having at least 20 nucleotides of any of the polynucleotides of the present invention and to polynucleotide sequences complementary to any of such polynucleotides or fragments.

[0014] In a preferred embodiment, the isolated polynucleotide is a strand of a full-length cDNA.

[0015] The present invention is further directed to isolated proteins encoded by any such full-length cDNA, as well as variants which have an amino acid sequence having at least 70% identity to such an isolated protein and retain the biological activity thereof, or biologically active fragments of such protein or variant, as well as to salts or functional derivatives of any such protein, variant or biologically active fragment.

[0016] The present invention is also directed to antibodies specific to any of the proteins, variants or fragments of the present invention and to any molecule which includes the antigen-binding portion of any such antibody.

[0017] The present invention also comprehends antisense DNA of a length sufficient to prevent transcription and/or translation of a gene identified in accordance with the present invention, as well as ribozymes which specifically bind and cleave mRNA sequences identified in accordance with the present invention.

[0018] The invention also comprehends methods for screening drugs which up-regulate or down-regulate a gene which is transcribed to an RNA containing a sequence of any of any of the polynucleotides of the present invention.

[0019] The present invention is additionally directed to pharmaceutical compositions which include the nucleic acids, proteins or polypeptides in accordance with the present invention, along with pharmaceutically acceptable carriers or excipients.

[0020] In addition, the present invention is directed to knockout or transgenic non-human animals, in which a gene identified by the present invention has been introduced or knocked out.

[0021] The present invention further provides a method of regulating angiogenesis or apoptosis in a patient in need of such treatment by administering to such patient a therapeutically effective amount of an antagonist of at least one protein as encoded by the nucleic acid sequences in accordance with the present invention.

[0022] Also provided is a diagnostic method for identifying genes modulated by hypoxic conditions by detecting the presence of a polynucleotide having a nucleic acid sequence according to the present invention.

[0023] Also provided is a method of regulating hypoxia-associated pathologies by administering an effective amount of at least one antisense oligonucleotide against one of the nucleic acid sequences (SEQ ID NOs:1-12) or their proteins. There is provided a method of regulating hypoxia associated

pathology by administering an effective amount of a protein encoded by the polynucleotides (SEQ ID NOs:1-12) as active ingredients in the pharmaceutically acceptable carrier.

[0024] Further, there are provided hypoxia response regulating genes.

[0025] Among the genes in accordance with the present invention is the novel gene 95, which shares homology with the PA26 gene. The mRNA levels of gene 95 are increased during hypoxia, regardless of the p53 status of the cells. In contrast, DNA damaging agents induce 95 expression in a p53-dependent manner. 95 is involved in regulation of cell survival under ischemia and hydrogen peroxide; however, it induces DNA damaged apoptosis. Conditioned medium from 95 overexpressing clones also possesses pro-apoptotic activity.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] Figure 1 shows sequence comparison of protein 95 (SEQ ID NO:4) and PA26 (SEQ ID NO:21).

[0027] Figure 2 is a graph showing how 95 overexpression affects the growth rate of proliferating breast tumor cells as compared to control clones in the presence or absence of tetracycline.

[0028] Figure 3 is a graph showing the effects of overexpression of 95 on MCF7 induced serum deprivation (0.1%) cell death. T is tetracyclie and s is serum.

[0029] Figure 4 is a graph showing the effects of 95 overexpression on protection of MCF7 cells against ischemia-induced cell death. T is tetracycline and I is ischemia.

[0030] Figure 5 is a graph showing the effects of 95 overexpression on protection of MCF7 cells against H_2O_2 (1 mM)-induced cell death. T is tetracycline and H is H_2O_2.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

I. Definitions

[0031] The following definitions apply to the terms used in the present specification and claims:

[0032] The term "gene" refers to the genomic nucleotide sequence which is transcribed to a full-length RNA. Such RNA molecules may be converted into corresponding cDNA molecules by techniques well known to the art of recombinant DNA technology. The term "gene" classically refers to the genomic sequence, which, upon processing, can produce different RNAs, e.g., by splicing events. However, for ease of reading, any full-length counterpart RNA sequence will also be referred to by shorthand herein as a "gene".

[0033] The term "Expressed Sequence Tag" or "EST" refers to a partial DNA or cDNA sequence of about 150 to 500, more preferably about 300, sequential nucleotides of a longer sequence obtained from a genomic or cDNA library prepared from a selected cell, cell type, tissue type, organ or organism

which longer sequence corresponds to an mRNA (or other full-length RNA) transcribed by a gene found in that library. In this case, the gene is found in rat neuronal cells. One or more libraries made from a single tissue type typically provide at least about 3,000 different (i.e., unique) ESTs and potentially the full complement of all possible ESTs representing all cDNAs, e.g., 50,000-100,000 in an animal such as a human. Further background and information on the construction of ESTs is described in Adams et al (1991) and International Application Number PCT/US92/05222 (January 7, 1993).

[0034] The term "apoptosis" is particularly defined as the single deletion of scattered cells by fragmentation into membrane-bound particles which are phagocytosed by other cells, believed to be due to programmed cell death. However, as used herein, it should be understood that this term should be construed more broadly as encompassing neuronal cell death, whether or not that cell death is strictly by means the apoptotic process described above.

[0035] Two proteins are "cognate" if they are produced in different species, but are sufficiently similar in structure and biological activity to be considered the equivalent proteins for those species. Two proteins may also be considered cognate if they have at least 50% amino acid

sequence identity (when globally aligned with a pam250 scoring matrix with a gap penalty of the form q+r(k-1) where k is the length of the gap, q=-12 and r=-4; percent identity=number of identities as percentage of length of shorter sequence) and at least one biological activity in common. Similarly, two genes are cognate if they are expressed in different species and encode cognate proteins.

II. Novel Polynucleotide Sequences

[0036] The present invention identifies polynucleotides (nucleic acid sequences) with sequences as set forth herein in SEQ ID NOs:1-12, that have been significantly up-regulated when subjected to hypoxia. SEQ ID NOs:1-4 and 8-12 have not previously been identified. SEQ ID NO 5 was found to match sequences in data banks but has not been reported to be associated with hypoxia regulation.

[0037] To the extent that the positively identified sequence is a novel sequence, the present invention comprehends that novel sequence, as well as any naturally-occurring polynucleotide that includes that sequence as a part thereof. The sequence *per se* has utility based on the fact that it has been identified on the basis of differential expression in cells subjected to hypoxic stress. It can be used in diagnostic processes and kits for determining whether any given cells have been subjected to hypoxic stress. Even

when such sequences are rat sequences, i.e., SEQ ID NOs:5 and 7, there is real-world utility for the purpose of medical research for determining in a rat model which cells have been subjected to hypoxic stress and which cells may have been protected from hypoxic stress when subjected to a treatment protocol in a rat model. By using the novel sequence as a probe, or a portion thereof as an oligonucleotide probe, one can identify the places in the organism (whether the organism is a rat when the sequence is a rat sequence or a human when the sequence is a human sequence) where the cDNA including the sequence is expressed and whether or not, or in what degree, it is expressed when subjected to various treatment protocols.

[0038] Human genes may be discovered by determining the human gene which corresponds to the rat gene discovered in accordance with the present invention. Such human genes are also useful for determining whether human cells have been subjected to hypoxic stress, for example in diagnosing whether or not a patient has suffered a stroke. As will be discussed in greater detail below, it is a procedurally routine matter to determine a cognate human gene based on the sequence of a rat gene. Thus, regardless of whether or not one knows the actual sequence of the corresponding human gene, the rat gene has utility as a probe for seeking and identifying the

corresponding human gene which, when identified, will have its own utility.

[0039] The positively identified polynucleotide sequences are ESTs. The location of an EST in a full-length cDNA is determined by analyzing the EST for the presence of coding sequence. A conventional computer program is used to predict the extent and orientation of the coding region of a sequence (using all six reading frames). Based on this information, it is possible to infer the presence of start or stop codons within a sequence and whether the sequence is completely coding or completely non-coding or a combination of the two. If start or stop codons are present, then the EST can cover both part of the 5'-untranslated or 3'-untranslated part of the mRNA (respectively) as well as part of the coding sequence. If no coding sequence is present, it is likely that the EST is derived from the 3' untranslated sequence due to its longer length and the fact that most cDNA library construction methods are biased toward the 3' end of the mRNA. It should be understood that both coding and non-coding regions may provide ESTs equally useful in the described invention.

[0040] As will be discussed below, even ESTs are directly useful as they have a length that allows for PCR (polymerase chain reaction), for use as a hybridization probe and have a

unique designation for the gene with which it hybridizes (generally under conditions sufficiently stringent to require at least 95% base pairing). For a detailed description and review of ESTs and their functional utility see, WO 93/00353 PCT Application which is incorporated herein in its entirety by reference, as well as the references by Zweiger et al, 1997; Okubo et al, 1997 and Braren et al, 1997.

[0041] The WO 93/00353 PCT application further describes how the EST sequences can be used to identify the transcribed genes.

[0042] Methods for obtaining complete gene sequences from ESTs are well-known to those of skill in the art. See, generally, Sambrook et al, (1989) and Ausubel et al (1994-2000). Briefly, one suitable method involves purifying the DNA from the clone that was sequenced to give the EST and labeling the isolated insert DNA. Suitable labeling systems are well known to those of skill in the art. See, e.g., Davis et al (1986). The labeled EST insert is then used as a probe to screen a lambda phage cDNA library or a plasmid cDNA library, identifying colonies containing clones related to the probe cDNA that can be purified by known methods. The ends of the newly purified clones are then sequenced to identify full-length sequences and complete sequencing of full-length clones is performed by enzymatic digestion or primer walking. A

similar screening and clone selection approach can be applied to clones from a genomic DNA library. The entire naturally-occurring cDNA or gene sequence, including any allelic variations thereof, all will have the same utility as discussed above for the identified polynucleotide.

[0043] The complete gene sequence of naturally-occurring variants of the gene in question, such as, for example, allelic variations, may be determined by hybridization of a cDNA library using a probe which is based on the identified polynucleotide, under highly stringent conditions or under moderately stringent conditions. Stringency conditions are a function of the temperature used in the hybridization experiment and washes, the molarity of the monovalent cations in the hybridization solution and in the wash solution(s) and the percentage of formamide in the hybridization solution. In general, sensitivity by hybridization with a probe is affected by the amount and specific activity of the probe, the amount of the target nucleic acid, the detectability of the label, the rate of hybridization, and the duration of the hybridization. The hybridization rate is maximized at a Ti (incubation temperature) of 20-25°C below Tm for DNA:DNA hybrids and 10-15°C below Tm for DNA:RNA hybrids. It is also maximized by an ionic strength of about 1.5M Na^+. The rate is

directly proportional to duplex length and inversely proportional to the degree of mismatching.

[0044] Specificity in hybridization, however, is a function of the difference in stability between the desired hybrid and "background" hybrids. Hybrid stability is a function of duplex length, base composition, ionic strength, mismatching, and destabilizing agents (if any).

[0045] The Tm of a perfect hybrid may be estimated for DNA:DNA hybrids using the equation of Meinkoth et al (1984), as

$$Tm = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% form) - 500/L$$

and for DNA:RNA hybrids, as

$$Tm = 79.8°C + 18.5 (\log M) + 0.58 (\%GC) - 11.8 (\%GC)^2 - 0.56(\% form) - 820/L$$

where

- M, molarity of monovalent cations, 0.01-0.4 M NaCl,
- %GC, percentage of G and C nucleotides in DNA, 30%-75%,
- % form, percentage formamide in hybridization solution, and
- L, length hybrid in base pairs.

[0046] Tm is reduced by 0.5-1.5°C (an average of 1°C can be used for ease of calculation) for each 1% mismatching.

[0047] The Tm may also be determined experimentally. As increasing length of the hybrid (L) in the above equations increases the Tm and enhances stability, the full-length rat gene sequence can be used as the probe.

[0048] Filter hybridization is typically carried out at 68°C, and at high ionic strength (e.g., 5-6 X SSC), which is non-stringent, and followed by one or more washes of increasing stringency, the last one being of the ultimately desired stringency. The equations for Tm can be used to estimate the appropriate Ti for the final wash, or the Tm of the perfect duplex can be determined experimentally and Ti then adjusted accordingly.

[0049] Hybridization conditions should be chosen so as to permit allelic variations, but avoid hybridizing to other genes. In general, stringent conditions are considered to be a Ti of 5°C below the Tm of a perfect duplex, and a 1% divergence corresponds to a 0.5-1.5°C reduction in Tm. Typically, rat clones were 95-100% identical to database rat sequences, and the observed sequence divergence may be artifactual (sequencing error) or real (allelic variation). Hence, use of a Ti of 5-15°C below, more preferably 5-10°C below, the Tm of the double stranded form of the probe is recommended for probing a rat cDNA library with rat EST

probes. However, when probing for a human gene cognate, more moderate stringency hybridization conditions should be used.

[0050] As used herein, highly stringent conditions are those which are tolerant of up to about 15% sequence divergence, while moderately stringent conditions are those which are tolerant of up to about 30-35% sequence divergence. Without limitation, examples of highly stringent (5-15°C below the calculated Tm of the hybrid) and moderately stringent (15-20°C below the calculated Tm of the hybrid) conditions use a wash solution of 0.1 X SSC (standard saline citrate) and 0.5% SDS at the appropriate Ti below the calculated Tm of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those which allow less stable hybrids to form along with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE), 5 X Denhardt's reagent, 0.5% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA at an appropriate incubation temperature Ti.

[0051] Once any such naturally-occurring DNA is identified, it can be tested by means of routine experimentation to

determine whether it is differentially expressed in the cells in which it naturally occurs when subjected to hypoxic stress. The present invention is intended to comprehend any such naturally-occurring DNA which binds to an EST of the present invention or any oligonucleotide fragment thereof, preferably having at least 20, more preferably at least 50, contiguous nucleic acids, under highly stringent conditions or under moderately stringent conditions, which identified DNA molecules are determined to be differentially expressed in the cells in which they naturally occur when such cells are subjected to hypoxic stress. Any such identified DNA molecules would have the same utility as discussed above for the identified polynucleotide.

[0052] If the full-length sequence identified is a rat gene sequence or a sequence of any mammalian gene other than human, the cognate human gene sequence can be readily obtained, as would be readily appreciated by those of skill in the art. Comparison of known cognate protein and gene sequences between rat and human shows a high level of sequence identity, mostly on the order of 70% or higher. The cognate human gene sequence is quite readily identified and determined as long as there is a high level of sequence identity to the rat gene sequence.

[0053] While a rat EST sequence would be used to probe a rat cDNA library for a full-length cDNA sequence, and could even be used to probe human cDNA libraries, it would be expected that there would be some sequence divergence, especially at the EST sequence level, between cognate rat and human DNAs, which sequence divergence may be possibly as much as 25-50%. Preferably, the rat sequence used as a probe is from the coding region of the rat cDNA, as 5'- or 3'-uncoded region often lack significant homology among different mammalian species.

[0054] If a partial human cDNA is obtained, it may be used to isolate a larger human cDNA, and the process repeated as needed until the complete human cDNA is obtained.

[0055] For cross-species hybridization, such as to obtain the cognate human gene sequence from the rat gene sequence, the Ti should be reduced further, by about 0.5-1.5°C, e.g., 1°C, for each expected 1% divergence in sequence. The degree of divergence may be estimated from the known divergence of the most closely related pairs of known genes from the two species.

[0056] If the desired degree of mismatching results in a wash temperature less than 45°C, it is desirable to increase the salt concentration so a higher temperature can be used. Doubling the SSC concentration results in about a 17°C

increase in Tm, so washes at 45°C in 0.1 X SSC and 62°C in 0.2 X SSC are equivalent (1 X SSC = 0.15 M NaCl, 0.015M trisodium citrate, pH 7.0).

[0057] The person skilled in the art can readily determine suitable combinations of temperature and salt concentration to achieve these degrees of stringency.

[0058] Examples of successful cross-species-hybridization experiments include Braun et al (1989) (mouse v. human), Imamura et al (1991) (human v. rat), Oro et al (1988) (human v. Drosophila), Higuti et al (1991) (rat v. human), Jeung et al (1992) (rat, bovine v. human), Iwata et al (1992) (human v. mouse), Libert et al (1992) (dog v. human), Wang et al (1993) (human v. mouse), Jakubiczka et al (1993) (human v. bovine), Nahmias et al (1991) (human v. mouse), Potier et al (1992) (rat v. human), Chan et al (1989) (human v. mouse), Hsieh et al (1989) (human, mouse v. bovine), Sumimoto et al (1989) (human v. mouse), Boutin et al (1989) (rat v. human), He et al (1990) (human, rat v. dog, guinea pig, frog, mouse), Galizzi et al (1990) (mouse v. human). See also Gould et al (1989).

[0059] In general, for cross-species hybridization, Ti = 25-35°C below Tm. Wash temperatures and ionic strengths may be adjusted empirically until background is low enough.

[0060] Any non-rat mammalian sequences obtained from such hybridization experiments, which sequences test positive for

the ability to be differentially expressed when the cells in which they naturally occur are subjected to hypoxic stress, are also encompassed by the present invention as are any non-human mammalian sequences obtained from such hybridization experiments using the human gene as a probe to find cognate non-human mammalian genes.

[0061] Fragments of any such naturally-occurring sequences also have utility and are intended to be encompassed by the present invention. Fragments of preferably at least 20, more preferably at least 50, nucleotides in length can be used as probes for the diagnostic assays described above.

[0062] Polynucleotide sequences that are complementary to any of the sequences or fragments encompassed by the present invention discussed above are also considered to be part of the present invention. Whenever any of the sequences discussed above are produced in a cell, the complementary sequence is concomitantly produced and, thus, the complementary sequence can also be used as a probe for the same diagnostic purposes.

[0063] Modifications or analogs of polynucleotides can be introduced to improve the therapeutic properties of the polynucleotides. Improved properties include increased nuclease resistance and/or increased ability to permeate cell membranes.

[0064] Nuclease resistance, where needed, is provided by any method known in the art that does not interfere with biological activity of the antisense oligodeoxy-nucleotides, cDNA and/or ribozymes as needed for the method of use and delivery (Iyer et al, 1990; Eckstein, 1985; Spitzer et al, 1988; Woolf et al, 1990; Shaw et al, 1991). Modifications that can be made to oligonucleotides in order to enhance nuclease resistance include modifying the phosphorous or oxygen heteroatom in the phosphate backbone. These include preparing methyl phosphonates, phosphorothioates, phosphorodithioates and morpholino oligomers. In one embodiment it is provided by having phosphorothioate bonds linking between the four to six 3'-terminus nucleotide bases. Alternatively, phosphorothioate bonds link all the nucleotide bases. Other modifications known in the art can be used where the biological activity is retained, but the stability to nucleases is substantially increased.

[0065] The present invention also includes all analogs of, or modifications to, a polynucleotide of the invention that does not substantially affect the function of the polynucleotide. The nucleotides can be selected from naturally occurring or synthetic modified bases. Naturally occurring bases include adenine, guanine, cytosine, thymine and uracil. Modified bases of the oligonucleotides include

xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiuracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thioalkyl guanines, 8-hydroxyl guanine and other substituted guanines, other aza and deaza adenines, other aza and deaza guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

[0066] In addition, analogs of nucleotides can be prepared wherein the structure of the nucleotide is fundamentally altered and that are better suited as therapeutic or experimental reagents. An example of a nucleotide analog is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in DNA (or RNA0 is replaced with a polyamide backbone which is similar to that found in peptides. PNA analogs have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. Further, PNAs have been shown to bind stronger to a complementary DNA sequence than a DNA molecule. This observation is attributed to the lack of charge repulsion between the PNA strand and the DNA strand. Other modifications that can be made to oligonucleotides include polymer backbones, cyclic backbones, or acyclic backbones.

III. Novel Proteins Encoded by Genes of Section II

[0067] Once the sequence of any full-length cDNA is obtained, the protein encompassed thereby is readily determinable by analysis of the sequence to find the start and stop codons and then decoding the amino acid sequence encoded by the cDNA. Thus, the present invention also encompasses any protein encoded by a full-length cDNA encompassed by the present invention as discussed above. Such proteins can be used for the same diagnostic utility, as discussed above for the polynucleotides, as they will be differentially expressed to the same degree that the corresponding cDNA is differentially expressed. They can be used to make a diagnostic tool which can be used to determine their presence in a cell. Thus, for example, they can be used to raise antibodies that could be used in such a diagnostic assay for the presence of such a protein. Such an assay would be useful to determine whether any given cell had been subjected to neurotoxic stress. Such proteins can also be used for any of the utilities discussed hereinbelow in the section related to methods of use.

[0068] Analogs of a protein or polypeptide encoded by the DNA sequences discovered in the assays described herein is also comprehended by the present invention. Preferably, the analog is a variant of the native sequence which has an amino

acid sequence having at least 70% identity to the native amino acid sequence and retains the biological activity thereof. More preferably, such a sequence has at least 85% identity, at least 90% identity, or most preferably at least 95% identity to the native sequence.

[0069] The term "sequence identity" as used herein means that the sequences are compared as follows. The sequences are aligned using Version 9 of the Genetic Computing Group's GAP (global alignment program), using the default (BLOSUM62) matrix (values -4 to +11) with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (per each additional consecutive null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the claimed sequence.

[0070] Analogs in accordance with the present invention may also be determined in accordance with the following procedure. Polypeptides encoded by any nucleic acid, such as DNA or RNA, which hybridize to the complement of the native DNA or RNA under highly stringent or moderately stringent conditions, as long as that polypeptide maintains the biological activity of the native sequence are also considered to be within the scope of the present invention. Preferably, such nucleic acids hybridizing to the complement of the polynucleotides of the

present invention under the specified conditions are naturally occurring nucleic acids, which may or may not be produced in cells of the same species as the original polynucleotides. As with any other analog, such polypeptide must retain the biological activity of the original polypeptide.

[0071] The term "active fragments" is intended to cover any fragment of the proteins identified by means of the present invention that retain the biological activity of the full protein. For example, fragments can be readily generated from the full protein where successive residues can be removed from either or both the N-terminus or C-terminus of the protein, or from biologically active peptides obtained therefrom by enzymatic or chemical cleavage of the polypeptide. Thus, multiple substitutions are not involved in screening for active fragments. If the removal of one or more amino acids from one end or the other does not affect the biological activity after testing in the standard tests, discussed herein, such truncated polypeptides are considered to be within the scope of the present invention. Further truncations can then be carried out until it is found where the removal of another residue destroys the biological activity.

[0072] "Functional derivatives" as used herein covers chemical derivatives which may be prepared from the functional

groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e., they do not destroy the biological activity of the corresponding protein as described herein and do not confer toxic properties on compositions containing it. Derivatives may have chemical moieties, such as carbohydrate or phosphate residues, provided such a fraction has the same biological activity and remains pharmaceutically acceptable.

[0073] Suitable derivatives may include aliphatic esters of the carboxyl of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives or free amino groups of the amino acid residues formed with acyl moieties (e.g., alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl group (e.g., that of seryl or threonyl residues) formed with acyl moieties. Such derivatives may also include for example, polyethylene glycol side-chains which may mask antigenic sites and extend the residence of the complex or the portions thereof in body fluids.

[0074] Non-limiting examples of such derivatives are described below.

[0075] Cysteinyl residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as

chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, alpha-bromo- beta-(5-imidazoyl)propionic acid, chloroacetyl phosphate, B alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl-2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4- nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

[0076] Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

[0077] Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2, 4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

[0078] Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3- butanedione, 1,2-cyclodexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

[0079] The specific modification of tyrosyl residues *per se* has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

[0080] Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-[2-morpholinyl-(4-ethyl)]carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethlypentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

[0081] Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl

residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

[0082] The term "derivatives" is intended to include only those derivatives that do not change one amino acid to another of the twenty commonly-occurring natural amino acids.

[0083] The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the complex of the invention or analogs thereof. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids, such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or oxalic acid. Of course, any such salts must have substantially similar biological activity to the complex of the invention or its analogs.

IV. Known Polynucleotides and Protein Sequences

[0084] To the extent that any of the polynucleotide sequences of the present invention are determined to appear in the sequence databanks and may be part of identified known

genes with known function and encode known proteins, it is not necessary to go through the hybridization steps in order to find the full-length cDNA for such ESTs. Furthermore, in most cases, it will not be necessary to find the cognate human gene experimentally. If the rat EST is part of a known rat gene, it is likely that the cognate human gene is also known. If not, it may be determined by the techniques discussed hereinabove with respect to novel rat gene sequences.

[0085] As the protein encoded by the known gene is also known, it is not necessary to use the techniques discussed hereinabove for determining the sequence encoded by a polynucleotide sequence. However, to the extent that the protein is not known, the techniques discussed hereinabove with respect to novel polynucleotide sequences may also be used.

[0086] Any known allelic variants of the known gene would also be expected to have the properties discovered by the gene discovery techniques discussed herein and, therefore, are also considered to be part of the present invention. The existence of other naturally-occurring variants having the property of having its sequence modulated when subjected to neurotoxic stress may also be determined using hybridization experiments under highly stringent conditions or moderately stringent

conditions, all as discussed in detail hereinabove with respect to the novel polynucleotide sequences.

[0087] Analogs, active fragments, functional derivatives and salts of the known proteins which retain the property of that protein for the purposes of the present invention (although not necessarily for the properties previously known for that protein) are comprehended by the present invention, if novel, and their use is considered to be part of the present invention.

V. Utility of Good Genes and Bad Genes

[0088] The genes found to be differentially expressed when the cell producing them are subjected to hypoxic stress, may be genes which contribute to the adverse effects of hypoxia, such as apoptosis, and, in some circumstances, angiogenesis, or genes which contribute to the alleviation of the detrimental effects of hypoxia. The former genes, which contribute to the adverse effects of hypoxia will be referred to as "bad genes" herein. It would be desirable to down-regulate or otherwise decrease the titre of the expression product of such bad genes at the site of the hypoxic event, such as stroke. The utility of such bad genes and methods of use thereof will be discussed below.

[0089] Those genes which contribute to the alleviation of the detrimental effects of hypoxia, including avoidance of

apoptosis and causing angiogenesis, will be referred to herein as "good genes". It would be desirable to up-regulate or otherwise increase the titres of the expression product of such good genes at the site of the hypoxic event. The utility of such good genes and methods of use thereof will be discussed below.

[0090] While it is not possible to directly determine from the differential expression studies in which tehse genes were found whether the DNA fragmenets found are part of a good or bad gene, it is reasonably certain that the fragments so identified are one or the other as their expression has been significantly modulated based on the hypoxic stress conditions to which the cells have been subjected. However, by means of further experimentation, which experimentation would not be considered to be undue experimentation, one can determine whether the fragments are part of good genes or bad genes. One way to test it is to create a mutation on the ATG codon of the fragment or create a frame shift mutation and then check whether its effect is the same. If the effect is different, it is a peptide which causes the effect. If the effect is the same, it is not a peptide but the RNA itself which causes the activity. Another possibility is to create a synthetic peptide and introduce it into cells to check whether it shows the relevant phenotype.

[0091] Another way to test whether the fragments are part of good genes or bad genes is to knock out the gene of interest, either in an animal with a knockout gene or by knocking out the gene in the cell line being tested. In a cell line, the cells can then be tested with hypoxic stress to determine whether the absence of that gene has a protective effect or enhances cell death. In a knockout mouse, similar tests can be conducted to see whether the absence of that gene has a protective or detrimental effect on the mouse when subjected to hypoxic stress.

[0092] A gene can be knocked out in a cell line by means of homologous recombination or by transfecting the cell line with an antisense sequence which prevents the expression of that gene, all as is well known to those of ordinary skill in this art. A gene can be knocked out in an animal such as a mouse, by the techniques discussed below.

[0093] Accordingly, even if it cannot be directly determined whether any of the specific DNA fragments of the present invention are parts of good genes or parts of bad genes, it is reasonably expected that they are parts of either one or the other, and, in either event, they have utility for the reasons discussed below. It can be determined whether they are good genes or bad genes without resorting to undue

experimentation. Accordingly, such genes have utility and industrial applicability.

[0094] Good genes are useful as the protein encoded by such genes can be used to protect neural from, and ameliorate the effects of, hypoxia and ischemia, and ultimately in the therapeutic treatment of stroke, hypoxia and/or ischemia. Such genes may prevent apoptosis or promote angiogenesis. As to the latter, promotion of angiogenesis may be desirable, for example, in trauma situations where a limb must be reattached or in a transplant where revascularization is needed. Thus the genes, and the DNA encoding such a protein or active fragment or analog thereof, are useful in the recombinant production of such proteins or polypeptides. They are also useful as a target for assays for the discovery of drugs which selectively up-regulate such genes. The proteins encoded by such novel good genes, as well as active fragments thereof, analogs and functional derivatives thereof, are also part of the present invention and have utility to protect cells from, and to ameliorate the effects of, hypoxia and ischemia, and ultimately in the therapeutic treatment of stroke, hypoxia ischemia, and/or other conditions where such effects would be desirable.

[0095] It may turn out that the beneficial effect of up-regulation of a good gene is due to the production of a non-

protein product of the gene's activity. Even in that case, however, up-regulation of the good gene will cause enhanced production of that product.

[0096] Good genes, whether novel or known, but whose relationship to hypoxia reported herein was previously unknown, may be used in novel processes which take advantage of these newly discovered properties. Thus, for example, the expression product of such genes, as well as active fragments, analogs and functional derivatives thereof, may be used to protect cells from the adverse effects of hypoxia or ischemia, to ameliorate the effects of hypoxia or ischemia, and ultimately for the treatment of the effects of stroke, hypoxia, ischemia, and/or other conditions where such effects would be desirable, by the therapeutic administration thereof in a manner which causes such product to be brought into the vicinity of the cells to be treated.

[0100] Bad genes are useful in that they can be used in diagnostic assays for cells that have been subjected to hypoxia or ischemia. If mRNA corresponding to such genes, or the translation product thereof, is found in the cells being assayed it is likely that they have been subjected to hypoxia or ischemia. If diagnosed pre-stroke, this may be predictive of incipient stroke. They are also useful as a target for assays for the discovery of drugs which selectively down-

regulate such genes or are otherwise dominant negative with respect to the expression of the gene product of such genes. Antisense RNA that prevents the expression of such gene is also part of the present invention and is useful to protect neural cells from neurotoxicity, to ameliorate the effects of hypoxia or ischemia, and ultimately for the treatment of the effects of stroke, hypoxia and/or ischemia. The bad gene may also be used therapeutically when these "bad" effects may be useful for treating a certain condition. For example, promotion of apoptosis may be useful for removing unwanted cells, such as tumor cells. Prevention of angiogenesis may also be useful under certain circumstances.

[0101] It may turn out that the detrimental effect of up-regulation of a bad gene is due to the production of non-protein product of the gene's activity. Even in that case, however, down-regulation of the bad gene will cause diminished production of that product.

[0102] Bad genes, whether novel or known but whose relationship to hypoxia reported herein was previously unknown, may be used in novel processes which take advantage of these newly discovered properties. Antisense RNA having a sequence complementary to a portion of such gene and that prevents the expression of such gene may be produced and used therapeutically by administering same in a manner by which it

enters cells which have been subjected to stroke, hypoxia, and/or ischemia in order to ameliorate the effects of such conditions. They may also be used in methods for assaying for drugs which down-regulate such genes. To the extent that such proteins are enzymes, the present invention comprehends the protection of neural cells from neurotoxicity, the amelioration of the effects of hypoxia or ischemia, and ultimately the therapeutic treatment of the effects of stroke, hypoxia and/or ischemia by administering an inhibitor of such enzyme in a manner that brings such inhibitor to the vicinity of the cells in which such enzyme has been up-regulated.

VI. Diagnostic Methods

[0103] As all of the genes of the present invention have been found to be modulated significantly upward after the cells have been subject to hypoxia, all of such genes may be considered to be a gene of interest for the purpose of the diagnostic assays reported herein.

[0097] Methods of detecting tissue hypoxia in mammalian tissue are based on the use of the mRNA of the genes of interest or the translation product thereof as a diagnostic marker for cells that have been subjected to hypoxia or ischemia. It is possible to determine the level of the mRNAs or protein translation products corresponding to these bad genes, in normal tissue or bodily fluids as compared to

hypoxic tissue a bodily fluid from a subject which has suffered a hypoxic event, and, thus, determine the reference values of these genes on mRNAs or proteins which are indicative of tissue hypoxia. For identification of the gene, *in situ* hybridization, Southern blotting, single strand conformational polymorphism, restriction endonuclease fingerprinting (REF), PCR amplification and DNA-chip analysis using the nucleic acid sequences of the present invention as probes/primers can be used.

[0104] Methods of obtaining tissue samples for analysis include any surgical and non-surgical technique known in the art. Surgical methods include, but are not limited to biopsy such as fine needle aspirate, core biopsy, dilation and curettage.

[0105] **Samples**. The sample for use in the detection methods may be of any biological fluid or tissue which is reasonably expected to contain the messenger RNA transcribed from one of the above genes of interest, or a protein expressed therefrom one of the above bad genes. The bodily fluids can include tears, serum, urine, sweat or other bodily fluid where secreted proteins from the tissue that is undergoing an ischemic event can be localized. Preferably, the sample is composed of cells from the subject being tested which are suspect of having been subjected to a hypoxic event,

such as neural cells from a suspected stroke area or cardiac cells from a suspect infarct area.

[0106] **Analyte Binding Reagents.** The assay target or analyte as a diagnostic marker may be a nucleic acid, such as mRNA of a gene of interest, or a protein translation product thereof. When the assay target is a nucleic acid, the preferred binding reagent is a complementary nucleic acid. However, the nucleic acid binding agent may also be a peptide or protein. A peptide phage library may be screened for peptides which bind the nucleic acid assay target. In a similar manner, a DNA binding protein may be randomly mutagenized in the region of its DNA recognition site, and the mutants screened for the ability to specifically bind the target. Or the hypervariable regions of antibodies may be mutagenized and the antibody mutants displayed on phage.

[0107] When the assay target is a protein, the preferred binding reagent is an antibody, the specifically binding fragment of an antibody, or a molecule that has the antigen-binding portion of an antibody. The antibody may be monoclonal or polyclonal. It can be obtained by first immunizing a mammal with the protein target, and recovering either polyclonal antiserum, or immunocytes for later fusion to obtain hybridomas, or by constructing an antibody phage library and screening the antibodies for binding to the

target. The binding reagent may also be a binding molecule other than an antibody, such as a receptor fragment, an oligopeptide, or a nucleic acid. A suitable oligopeptide or nucleic acid may be identified by screening a suitable random library.

[0108] **Signal Producing System (SPS).** In order to detect the presence, or measure the amount, of an analyte, the assay must provide for a signal producing system (SPS) in which there is a detectable difference in the signal produced, depending on whether the analyte is present or absent (or, in a quantitative assay, on the amount of the analyte). The detectable signal may be one which is visually detectable, or one detectable only with instruments. Possible signals include production of colored or luminescent products, alteration of the characteristics (including amplitude or polarization) of absorption or emission of radiation by an assay component or product, and precipitation or agglutination of a component or product. The term "signal" is intended to include the discontinuance of an existing signal, or a change in the rate of change of an observable parameter, rather than a change in its absolute value. The signal may be monitored manually or automatically.

[0109] **Labels.** The component of the signal producing system which is most intimately associated with the diagnostic

reagent for the analyte is called the "label". A label may be, e.g., a radioisotope, a fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, an agglutinable particle, etc.

[0110] The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are ^{3}H, ^{32}P, ^{125}I, ^{131}I, ^{35}S, and ^{14}C.

[0111] Diagnostic kits are also within the scope of this invention. Such kits include monoclonal antibodies or nucleic acid probes that can rapidly detect tissue hypoxia.

[0112] For nucleic acid probes, the radioactive labeling can be carried out according to any conventional method such as terminal labeling at the 3' or 5' position with the use of a radiolabeled nucleotide, a polynucleotide kinase (with or without dephosphorylation by a phosphatase) or a ligase (according to the extremity to be labeled). The probes can be the matrix for the synthesis of a chain consisting of several radioactive nucleotides or of several radioactive and non-radioactive nucleotides. The probes can also be prepared by a chemical synthesis using one or several radioactive nucleotides. Another method for radioactive labeling is a

chemical iodination of the probes of the invention which leads to the binding of several ^{125}I atoms on the probes.

[0113] The label may also be a fluorophore. When the fluorescently labeled reagent is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

[0114] Alternatively, fluorescence-emitting metals such as ^{125}Eu, or others of the lanthanide series, may be incorporated into a diagnostic reagent using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) of ethylenediamine-tetraacetic acid (EDTA).

[0115] The label may also be a chemiluminescent compound. The presence of the chemiluminescently labeled reagent is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isolumino, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

[0116] Likewise, a bioluminescent compound may be used for labeling. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein

increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

[0117] Enzyme labels, such as horseradish peroxidase and alkaline phosphatase, can also be used. When an enzyme label is used, the signal producing system must also include a substrate for the enzyme. If the enzymatic reaction product is not itself detectable, the SPS will include one or more additional reactants so that a detectable product appears.

[0118] **Conjugation Methods**. A label may be conjugated, directly or indirectly (e.g., through a labeled anti-analyte binding reagent antibody), covalently (e.g., with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)) or non-covalently, to the analyte binding reagent, to produce a diagnostic reagent.

[0119] Similarly, the analyte binding reagent may be conjugated to a solid phase support to form a solid phase ("capture") diagnostic reagent.

[0120] Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to

some extent or insoluble for the purposes of the present invention.

[0121] The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to its target. Thus the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.

[0122] **Binding Assay Formats.** Binding assays may be divided into two basic types, heterogeneous and homogeneous. In heterogeneous assays, the interaction between the affinity molecule and the analyte does not affect the label, hence, to determine the amount or presence of analyte, bound label must be separated from free label. In homogeneous assays, the interaction does affect the activity of the label, and therefore analyte levels can be deduced without the need for a separation step.

[0123] In one embodiment, the analyte binding reagent is insolubilized by coupling it to a macromolecular support, and analyte in the sample is allowed to compete with a known quantity of a labeled or specifically labelable analyte analogue. The "analyte analogue" is a molecule capable of competing with analyte for binding to the analyte binding

reagent, and the term is intended to include analyte itself. It may be labeled already, or it may be labeled subsequently by specifically binding the label to a moiety differentiating the analyte analogue from analyte. The solid and liquid phases are separated, and the labeled analyte analogue in one phase is quantified. The higher the level of analyte analogue in the solid phase, i.e., sticking to the analyte binding reagent, the lower the level of analyte in the sample.

[0124] In a "sandwich assay", both an insolubilized analyte binding reagent, and a labeled analyte binding reagent are employed. The analyte is captured by the insolubilized analyte binding reagent and is tagged by the labeled analyte binding reagent, forming a ternary complex. The reagents may be added to the sample in either order, or simultaneously. The analyte binding reagents may be the same or different. The amount of labeled analyte binding reagent in the ternary complex is directly proportional to the amount of analyte in the sample.

[0125] The two embodiments described above are both heterogeneous assays. However, homogeneous assays are conceivable. The key is that the label be affected by whether or not the complex is formed.

[0126] **Detection of Genes of Interest.** Detection of the mRNA of the genes of interest may be done by Northern blot

analysis on tissue biopsies. Tissue samples from patients may be obtained and the total RNA extracted using RNAStat 60. The total RNA sample may then be resolved on denaturing gel by electrophoresis and then transferred onto a nylon membrane. After transfer of RNA onto the membrane, the membrane may then be used in hybridization with a suitable probe, which may be a synthetic probe directed against a gene already known to be a marker, or which may be a cDNA probe prepared directly from subtractive hybridization, wherein the fragment encoding the gene of interest, that is up-regulated in tissue hypoxia, will be labeled, preferably either radioactively with ^{32}P or non-radioactively with DIG (Digoxigenin). A negative control, such as one composed of RNA sample from normal tissue of normal subjects, may be resolved side by side with the patients' sample, to determine quantitatively whether there is a significant increase in the level of gene expression. Elevation of the messenger RNA transcript from this gene would imply the presence of hypoxia, ischemia or other neurotoxic stress.

[0127] In a hybridization assay, a nucleic acid reagent is used as a probe. For probe use, only one reagent is needed, and it may hybridize to all or just a part of the target nucleic acid. Optionally, more than one probe may be used to increase specificity.

[0128] In probe-based assays, hybridizations may be carried out on filters or in solutions. Typical filters are nitrocellulose, nylon, and chemically-activated papers. The probe may be double stranded or single stranded, however, the double stranded nucleic acid will be denatured for binding.

[0129] Techniques for detecting a protein translation product of interest include, but are not limited to, immunoblotting or Western blotting, ELISA, sandwich assays, fluorescence, or biotin or enzymatic labeling with or without secondary antibodies.

[0130] Western blot analysis can be done on the tissue biopsies or tissue aspirates. This would involve resolving the proteins on an electrophoretic gel, such as an SDS PAGE gel, and transferring the resolved proteins onto a nitrocellulose or other suitable membrane. The proteins are incubated with a target binding molecule, such as an antibody.

[0131] This binding reagent may be labeled or not. If it is unlabeled, then one would also employ a secondary, labeled molecule which binds to the binding reagent. One approach involves avidinating one molecule and biotinylating the other. Another is for the secondary molecule to be a secondary antibody which binds the original binding reagent.

[0132] To improve detection of the specific protein, immunoprecipitation can be conducted. This typically will

involve addition of a monoclonal antibody against the protein of interest to samples, then allowing the Ig-protein complex to precipitate after the addition of an affinity bead (ie antihuman Ig Sepharose bead). The immunoprecipitates will undergo several washings prior to transfer onto a nitrocellulose membrane. The Western blot analysis can be performed using another antibody against the primary antibody used.

[0133] There are a number of different methods of delivering the radiolabeled analyte binding reagent to the end-user in an amount sufficient to permit subsequent dynamic and/or static imaging using suitable radiodetecting devices. It may be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. Because proteins and nucleic acids are subject to being digested when administered orally, parenteral administration, i.e., intravenous, subcutaneous, orintramuscular, would ordinarily be used to optimize absorption of an analyte binding reagent, such as an antibody, which is a protein.

[0134] The dosage is the smallest amount capable of providing a diagnostically effective image, and may be determined by means conventional in the art, using known radioimaging agents as a guide.

[0135] Typically, the imaging is carried out on the whole body of the subject, or on that portion of the body or organ relevant to the condition or disease under study. The amount of radiolabeled analyte binding reagent accumulated at a given point in time in relevant target organs can then be quantified.

[0136] A particularly suitable radiodetecting device is a scintillation camera, such as a gamma camera. A scintillation camera is a stationary device that can be used to image distribution of radiolabeled analyte binding reagent. The detection device in the camera senses the radioactive decay, the distribution of which can be recorded. Data produced by the imaging system can be digitized. The digitized information can be analyzed over time discontinuously or continuously. The digitized data can be processed to produce images, called frames, of the pattern of uptake of the radiolabeled analyte binding reagent in the target tissue/organ at a discrete point in time. In most continuous (dynamic) studies, quantitative data is obtained by observing changes in distributions of radioactive decay in the target tissue/organ over time. In other words, a time-activity analysis of the data will illustrate uptake through clearance of the radiolabeled binding protein by the target organs with time.

[0137] Various factors should be taken into consideration in selecting an appropriate radioisotope. The radioisotope must be selected with a view to obtaining good quality resolution upon imaging, should be safe for diagnostic use in humans and animals (except for animal models which will be sacrificed thereafter and will be maintained anaesthetized until then), and should preferably have a short physical half-life so as to decrease the amount of radiation received by the body (with the same exceptions). The radioisotope used should preferably be pharmacologically inert, and, in the quantities administered, should not have any substantial physiological effect.

[0138] The analyte binding reagent may be radiolabeled with different isotopes of iodine, for example ^{123}I, ^{125}I, or ^{131}I (see for example, U.S. Patent 4,609,725). The extent of radiolabeling must, however be monitored, since it will affect the calculations made based on the imaging results (i.e., a diiodinated analyte binding reagent will result in twice the radiation count of a similar monoiodinated analyte binding reagent over the same time frame).

[0139] In applications to human subjects, it may be desirable to use radioisotopes other than ^{125}I for labeling in order to decrease the total dosimetry exposure of the human body and to optimize the detectability of the labeled molecule

(though this radioisotope can be used if circumstances require). Ready availability for clinical use is also a factor. Accordingly, for human applications, preferred radiolabels are for example, ^{99m}Tc, ^{67}Ga, ^{68}Ga, ^{90}Y, ^{111}In, ^{113m}In, ^{123}I, ^{186}Re, ^{188}Re or ^{211}At.

[0140] The radiolabeled analyte binding reagent may be prepared by various methods. These include radiohalogenation by the chloramine-T method or the lactoperoxidase method and subsequent purification by HPLC (high pressure liquid chromatography), for example as described by Gutkowska et al (1987). Other known method of radiolabeling can be used, such as IODOBEADS™.

[0141] For animal models, such as mice or rats, the animal may be sacrificed after administration of the analyte binding reagent and regions which have been subjected to neurotoxic stress imaged on immobilized brain slices.

VII. Screening Methods

[0142] Each of the genes identified by means of the present invention can be used as a candidate gene in a screening assay for identifying and isolating inhibitors of hypoxia or other neurotoxic stress. Many types of screening assays are known to those of ordinary skill in the art. The specific assay which is chosen will depend to a great extent on the activity of the candidate gene or the protein expressed thereby. Thus,

if it is known that the expression product of a candidate gene has enzymatic activity, then an assay which is based on inhibition of the enzymatic activity may be used. If the candidate protein is known to bind to a ligand or other interactor, then the assay can be based on the inhibition of such binding or interaction. When the candidate gene is a known gene, then many of its properties will also be known, and these can be used to determine the best screening assay. If the candidate gene is novel, then some analysis and/or experimentation will be appropriate in order to determine the best assay to be used to find inhibitors of the activity of that candidate gene. The analysis may involve a sequence analysis to find domains in the sequence which would shed light on its activity. Other experimentation described herein to identify the candidate gene and its activity, which experiment would not amount to undue experimentation, may also be engaged in so as to identify the type of screen that would be appropriate to find inhibitors or enhancers, as the case may be, for the candidate gene or the protein encoded thereby.

[0143] As is well known in the art, the screening assays may be *in vivo* or *in vitro*. An *in vivo* assay is a cell-based assay using any eukaryotic cell. One such cell-based system is particularly relevant in order to directly measure the activity of candidate genes which are pro-apoptotic functional

genes, i.e., expression of the gene will cause apoptosis or otherwise cause cell death in target cells. One way of running such an *in vivo* assay uses tetracycline-inducible (Tet-inducible) gene expression. Tet-inducible gene expression is well known in the art (Hofmann et al, 1996). Tet-inducible retroviruses have been designed incorporating the Self-inactivating (SIN) feature of a 3' Ltr enhancer/promoter retroviral deletion mutant. Expression of this vector in cells is virtually undetectable in the presence of tetracycline or other active analogs. However, in the absence of Tet, expression is turned on to maximum within 48 hours after induction, with uniform increased expression of the whole population of cells that harbor the inducible retrovirus, indicating that expression is regulated uniformly within the infected cell population.

[0144] When dealing with pro-apoptotic function candidate genes, Tet-inducible expression causes apoptosis in target cells. One can screen for small molecules or peptides able to rescue the cells from the gene-triggered apoptosis.

[0145] If the gene product of the candidate gene phosphorylates with a specific target protein, a specific reporter gene construct can be designed such that phosphorylation of this reporter gene product causes its activation, which can be followed by a color reaction. The

candidate gene can be specifically induced, using the Tet-inducible system discussed above, and a comparison of induced vs. non-induced genes provides a measure of reporter gene activation.

[0146] In a similar indirect assay, a reporter system can be designed that responds to changes in protein-protein interaction of the candidate protein. If the reporter responds to actual interaction with the candidate protein, a color reaction will occur.

[0147] One can also measure inhibition or stimulation of reporter gene activity by modulation of its expression levels via the specific candidate promoter or other regulatory elements. A specific promoter or regulatory element controlling the activity of a candidate gene is defined by methods well known in the art. A reporter gene is constructed which is controlled by the specific candidate gene promoter or regulatory elements. The DNA containing the specific promoter or regulatory agent is actually linked to the gene encoding the reporter. Reporter activity depends on specific activation of the promoter or regulatory element. Thus, inhibition or stimulation of the reporter will be a direct assay of stimulation/inhibition of the reporter gene.

[0148] Various *in vitro* screening assays are also well within the skill of those of ordinary skill in the art. For

example, if enzymatic activity is to be measured, such as if the candidate protein has a kinase activity, the target protein can be defined and specific phosphorylation of the target can be followed. The assay may involve either inhibition of target phosphorylation or stimulation of target phosphorylation, both types of assay being well known in the art.

[0149] One can also measure *in vitro* interaction of a candidate protein with interactors. In this screen, the candidate protein is immobilized on beads. An interactor, such as a receptor ligand, is radioactively labeled and added. When it binds to the candidate protein on the bead, the amount of radioactivity carried on the beads (due to interaction with the candidate protein) can be measured. The assay would indicate inhibition of the interaction by measuring the amount of radioactivity on the bead.

[0150] Any of the screening assays, according to the present invention, will include a step of identifying the small molecule or peptide which tests positive in the assay and may also include the further step of producing that which has been so identified. The use of any such molecules identified for inhibiting hypoxia or other neurotoxic stress is also considered to be part of the present invention.

VIII. Therapeutic Methods Relating to Good Genes

[0151] In accordance with these findings, the present invention extends to the treatment of stroke by the administration of a stroke-ameliorating or stroke-inhibiting amount of an agent capable of at least partially preventing brain damage, or averting the occurrence or reducing the size and severity of an ischemic infarct due, for example, to stroke, aneurysm, cerebrovascular accident, apoplexy or other trauma. Other conditions in which apoptosis is to be prevented or angiogenesis promoted may also be treatable by administration of the good genes of the present invention. Exemplary situations where a promoter of angiogenesis would be useful include trauma situations where a limb must be reattached or in a transplant where revascularization is needed.

[0152] The present invention, therefore, extends to methods for the treatment of stroke or other conditions caused or exacerbated by hypoxia or ischemia as where apoptosis is to be prevented or angiogenesis promoted, and to corresponding pharmaceutical compositions, comprising and including, without limitation, as active ingredients a protein encoded by a good gene, as well as analogs, active fragments, functional derivatives or salts thereof.

[0153] Within minutes after cessation of local cerebral blood flow, a region of densely ischemic brain tissue becomes infarcted and dies. This infarcted core is surrounded however, by a zone of ischemic but potentially viable tissue termed the "ischemic penumbra," which receives suboptimal perfusion via collateral blood vessels. The volume of the penumbra that ultimately becomes infarcted after an acute arterial occlusion is determined by a variety of factors that mediate neurotoxicity within this zone during the hours following interrupted blood flow. The nature of these factors (including glutamate, superoxide radicals, and nitric oxide) is only partially understood, as are the complex interactions that will determine whether ischemic tissue will die or recover. Some of these factors are intrinsic to the locus of ischemia, and others are delivered to the penumbra via the circulation. The net result of signaling interactions between these factors can greatly enhance neuronal cytotoxicity in the ischemic penumbra, causing a significantly larger volume of brain damage and necrosis, with corresponding increases in functional damage. The good genes, in accordance with the present invention, participate in mediating increased volumes of cerebral infarction during focal cerebral ischemia.

[0154] Good genes may also be used as the target of screening processes to find agents capable of enhancing the

expression of a good gene. Thus, the amount of mRNA produced by a cell, before and after subjecting the cell to a neurotoxic stress, such as hypoxia, and administering a test agent, will determine whether that test agent causes further enhancement of expression of that good gene, as compared to a control in which no test agent is added. Such testing can reveal agents which are useful in the treatment of stroke. Screening methods are discussed in Section VII, hereinabove.

IX. Therapeutic Methods Relating to Bad Genes

[0155] Bad genes may be used therapeutically for treating conditions in which promotion of apoptosis and/or inhibition of angiogenesis is desirable. Promotion of apoptosis would be useful in treating tumor cells. Inhibition of angiogenesis may be useful, for example, with vascular stents where ingrowth is undesirable. The present invention, therefore, extends to methods for the treatment of cancer and other conditions where promotion of apoptosis and/or inhibition of angiogenesis is desired, and to corresponding pharmaceutical compositions, comprising and including, without limitation, as active ingredients a protein encoded by a bad gene, as well as analogs, active fragments, functional derivatives or salts thereof.

[0156] Additionally, the ability of an agent to inhibit expression of bad genes provides an additional therapeutic

mechanism in the treatment of stroke since it would be expected to result in a reduction in the size and severity of the infarction.

[0157] The present invention thus includes a method of screening for an agent capable of providing a neuroprotective effect and thus reducing the size and severity of infarct size in stroke, which method comprises administering a test agent concurrent with, or subsequent to, an infarct-producing amount of a product of a bad gene and measuring the resultant decrease in infarct size vis-a-vis a control dose of the infarct-producing amount of the polyamine. Such testing can reveal agents which are useful in the treatment of this aspect of stroke. Screening methods are discussed in Section VII, hereinabove.

[0158] The production and administration of antisense sequences and ribozymes that specifically bind and cleave a particular mRNA sequence are discussed in Sections XI and XII hereinafter. Such ribozymes and antisense sequences relating specifically to bad genes and the mRNA they describe will inhibit the expression of these bad genes and, thus, will provide an additional therapeutic mechanism in treating the effects of stroke, hypoxia and/or ischemia or other conditions in which apoptosis is to be inhibited and/or angiogenesis promoted. Similarly, negative dominant peptides are discussed

in Section XIII. Such negative dominant peptides relating specifically to bad genes will inhibit the expression of these bad genes or the effects of the gene product of such bad genes and, thus, will provide yet another therapeutic mechanism in treating the effects of stroke, hypoxia and/or ischemia or other conditions in which apoptosis is to be inhibited and/or angiogenesis promoted.

X. Antibodies

[0159] The present invention also comprehends antibodies specific for the proteins encoded by a naturally-occurring cDNA which is part of the present invention as discussed above. Such an antibody may be used for diagnostic purposes to identify the presence of any such naturally-occurring proteins. Such antibody may be a polyclonal antibody or a monoclonal antibody or any other molecule that incorporates the antigen-binding portion of a monoclonal antibody specific for such a protein. Such other molecules may be a single-chain antibody, a humanized antibody, an F(ab) fraction, a chimeric antibody, an antibody to which is attached a label, such as fluorescent or radioactive label, or an immunotoxin in which a toxic molecule is bound to the antigen binding portion of the antibody. The examples are intended to be non-limiting. However, as long as such a molecule includes the antigen-binding portion of the antibody, it will be expected

to bind to the protein and, thus, can be used for the same diagnostic purposes for which a monoclonal antibody can be used.

[0160] Conveniently, the antibodies can be prepared against the immunogen or portion thereof for example a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof can be isolated and used as the immunogen. Immunogens can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow et al (1988) and Borrebaeck (1992). Antibody fragments can also be prepared from the antibodies and include Fab, $F(ab')_2$, and Fv by methods known to those skilled in the art.

[0161] For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the immunogen or immunogen fragment, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the immunogen are collected from the sera. Further, the polyclonal antibody can be absorbed such that it is monospecific. That is, the sera can be absorbed against related immunogens so that no cross-reactive antibodies remain in the sera rendering it monospecific.

[0162] For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the immunogen, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

[0163] For producing recombinant antibody (see generally Huston et al, 1991; Johnson et al, 1991; Mernaugh et al, 1995), messenger RNAs from antibody producing B-lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complimentary DNAs (cDNAs). Antibody cDNA, which can be full or partial length, is amplified and cloned into a phage or a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The antibody, or antibody fragment, is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

[0164] The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated, as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic

moieties see Johnstone et al, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (See for a general discussion Harlow et al, 1988, and Borrebaeck, 1992). The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, β-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

XI. Antisense Sequences

[0165] In order to manipulate the expression of a bad gene, it is desirable to produce antisense RNA in a cell. To this end, the complete or partial cDNA of a bad gene in accordance with the present invention is inserted into an expression vector comprising a promoter. The 3' end of the cDNA is thereby inserted adjacent to the 3' end of the promoter, with the 5' end of the cDNA being separated from the 3' end of the promoter by said cDNA. Upon expression of the cDNA in a cell, an antisense RNA is therefore produced which is incapable of coding for the protein. The presence of antisense RNA in the cell reduces the expression of the cellular (genomic) copy of the bad gene.

[0166] For the production of antisense RNA, the complete cDNA may be used. Alternatively, a fragment thereof may be

used, which is preferably between about 9 and 2,000 nucleotides in length, more preferably between 15 and 500 nucleotides, and most preferably between 30 and 150 nucleotides.

[0167] The fragment is preferably corresponding to a region within the 5' half of the cDNA, more preferably the 5' region comprising the 5' untranslated region and/or the first exon region, and most preferably comprising the ATG translation start site. Alternatively, the fragment may correspond to DNA sequence of the 5' untranslated region only.

[0168] Antisense intervention in the expression of specific genes can be achieved by the use of synthetic AS oligonucleotide sequences (for recent reports see Lefebvre-d'Hellencourt et al, 1995; Agrawal, 1996; Lev-Lehman et al, 1997). The oligonucleotide is preferably a DNA oligonucleotide. The length of the antisense oligonucleotide is preferably between 9 and 150, more preferably between 12 and 60, and most preferably between 15 and 50 nucleotides. Suitable antisense oligonucleotides that inhibit the production of the protein of the present invention from its encoding mRNA can be readily determined with only routine experimentation through the use of a series of overlapping oligonucleotides similar to a "gene walking" technique that is well-known in the art. Such a "walking" technique as well-

known in the art of antisense development can be done with synthetic oligonucleotides to walk along the entire length of the sequence complementary to the mRNA in segments on the order of 9 to 150 nucleotides in length. This "gene walking" technique will identify the oligonucleotides that are complementary to accessible regions on the target mRNA and exert inhibitory antisense activity.

[0169] The AS oligonucleotide sequence is designed to complement a target mRNA of interest and form an RNA:AS duplex. This duplex formation can prevent processing, splicing, transport or translation of the relevant mRNA. Moreover, certain AS nucleotide sequences can elicit cellular RNase H activity when hybridized with their target mRNA, resulting in mRNA degradation (Calabretta et al, 1996). In that case, RNase H will cleave the RNA component of the duplex and can potentially release the AS to further hybridize with additional molecules of the target RNA. An additional mode of action results from the interaction of AS with genomic DNA to form a triple helix which can be transcriptionally inactive.

[0170] The sequence target segment for the antisense oligonucleotide is selected such that the sequence exhibits suitable energy related characteristics important for oligonucleotide duplex formation with their complementary templates, and shows a low potential for self-dimerization or

self-complementation (Anazodo et al, 1996). For example, the computer program OLIGO 4.0 (National Biosciences, Inc.), can be used to determine antisense sequence melting temperature, free energy properties, and to estimate potential self-dimer formation and self-complimentary properties. The program allows the determination of a qualitative estimation of these two parameters (potential self-dimer formation and self-complimentary) and provides an indication of "no potential" or "some potential" or "essentially complete potential". Using this program target segments are generally selected that have estimates of no potential in these parameters. However, segments can be used that have "some potential" in one of the categories. A balance of the parameters is used in the selection as is known in the art. Further, the oligonucleotides are also selected as needed so that analog substitution do not substantially affect function.

[0171] Alternatively, an oligonucleotide based on the coding sequence of a protein capable of binding to a bad gene or the protein encoded thereby can be designed using Oligo 4.0 (National Biosciences, Inc.). Antisense molecules may also be designed to inhibit translation of an mRNA into a polypeptide by preparing an antisense which will bind in the region spanning approximately -10 to +10 nucleotides at the 5' end of the coding sequence.

[0172] The mechanism of action of antisense RNA and the current state of the art on use of antisense tools is reviewed in Kumar et al (1998). There are reviews on the chemical (Crooke, 1995; Uhlmann et al, 1990), cellular (Wagner, 1994) and therapeutic (Hanania, et al, 1995; Scanlon, et al, 1995; Gewirtz, 1993) aspects of this rapidly developing technology. The use of antisense oligonucleotides in inhibition of BMP receptor synthesis has been described by Yeh et al (1998). The use of antisense oligonucleotides for inhibiting the synthesis of the voltage-dependent potassium channel gene Kv1.4 has been described by Meiri et al (1998). The use of antisense oligonucleotides for inhibition of the synthesis of Bcl-x has been described by Kondo et al (1998). The therapeutic use of antisense drugs is discussed by Stix (1998); Flanagan (1998); Guinot et al (1998), and references therein. Within a relatively short time, ample information has accumulated about the *in vitro* use of AS nucleotide sequences in cultured primary cells and cell lines as well as for *in vivo* administration of such nucleotide sequences for suppressing specific processes and changing body functions in a transient manner. Further, enough experience is now available *in vitro* and *in vivo* in animal models and human clinical trials to predict human efficacy.

[0173] Modifications of oligonucleotides that enhance desired properties are generally used when designing antisense oligonucleotides. For instance, phosphorothioate bonds are used instead of the phosphoester bonds that naturally occur in DNA, mainly because such phosphorothioate oligonucleotides are less prone to degradation by cellular enzymes. Peng Ho et al teach that undesired *in vivo* side effects of phosphorothioate oligonucleotides may be reduced when using a mixed phosphodiester-phosphorothioate backbone. Preferably, 2'-methoxyribonucleotide modifications in 60% of the oligonucleotide is used. Such modified oligonucleotides are capable of eliciting an antisense effect comparable to the effect observed with phosphorothioate oligonucleotides. Peng Ho et al teach further that oligonucleotide analogs incapable of supporting ribonuclease H activity are inactive.

[0174] Therefore, the preferred antisense oligonucleotide of the present invention has a mixed phosphodiester-phosphorothioate backbone. Preferably, 2'-methoxyribonucleotide modifications in about 30% to 80%, more preferably about 60%, of the oligonucleotide are used.

[0175] In the practice of the invention, antisense oligonucleotides or antisense RNA may be used. The length of the antisense RNA is preferably from about 9 to about 3,000 nucleotides, more preferably from about 20 to about 1,000

nucleotides, most preferably from about 50 to about 500 nucleotides.

[0176] In order to be effective, the antisense oligonucleotides of the present invention must travel across cell membranes. In general, antisense oligonucleotides have the ability to cross cell membranes, apparently by uptake via specific receptors. As the antisense oligonucleotides are single-stranded molecules, they are to a degree hydrophobic, which enhances passive diffusion through membranes. Modifications may be introduced to an antisense oligonucleotide to improve its ability to cross membranes. For instance, the oligonucleotide molecule may be linked to a group which includes partially unsaturated aliphatic hydrocarbon chain and one or more polar or charged groups such as carboxylic acid groups, ester groups, and alcohol groups. Alternatively, oligonucleotides may be linked to peptide structures, which are preferably membranotropic peptides. Such modified oligonucleotides penetrate membranes more easily, which is critical for their function and may, therefore, significantly enhance their activity. Palmityl-linked oligonucleotides have been described by Gerster et al (1998). Geraniol-linked oligonucleotides have been described by Shoji et al (1998). Oligonucleotides linked to peptides, e.g., membranotropic peptides, and their preparation have been

described by Soukchareun et al (1998). Modifications of antisense molecules or other drugs that target the molecule to certain cells and enhance uptake of the oligonucleotide by said cells are described by Wang (1998).

[0177] The antisense oligonucleotides of the invention are generally provided in the form of pharmaceutical compositions. These compositions are for use by injection, topical administration, or oral uptake.

[0178] Preferred uses of the pharmaceutical compositions of the invention by injection are subcutaneous injection, intraperitoneal injection, and intramuscular injection.

[0179] The pharmaceutical composition of the invention generally comprises a buffering agent, an agent which adjusts the osmolarity thereof, and optionally, one or more carriers, excipients and/or additives as known in the art, e.g., for the purposes of adding flavors, colors, lubrication, or the like to the pharmaceutical composition.

[0180] Carriers may include starch and derivatives thereof, cellulose and derivatives thereof, e.g., microcrystalline cellulose, Xanthum gum, and the like. Lubricants may include hydrogenated castor oil and the like.

[0181] A preferred buffering agent is phosphate-buffered saline solution (PBS), which solution is also adjusted for osmolarity.

[0182] A preferred pharmaceutical formulation is one lacking a carrier. Such formulations are preferably used for administration by injection, including intravenous injection.

[0183] The preparation of pharmaceutical compositions is well known in the art and has been described in many articles and textbooks, see e.g., Remington's Pharmaceutical Sciences, especially pp 1521-1712 therein (Gennaro, 1990).

[0184] Additives may also be selected to enhance uptake of the antisense oligonucleotide across cell membranes. Such agents are generally agents that will enhance cellular uptake of double-stranded DNA molecules. For instance, certain lipid molecules have been developed for this purpose, including the transfection reagents DOTAP (Boehringer Mannheim), Lipofectin, Lipofectam, and Transfectam, which are available commercially. For a comparison of various of these reagents in enhancing antisense oligonucleotide uptake, see e.g., Quattrone et al (1995) and Capaccioli et al (1993). The antisense oligonucleotide of the invention may also be enclosed within liposomes. The preparation and use of liposomes, e.g., using the above-mentioned transfection reagents, is well known in the art. Other methods of obtaining liposomes include the use of Sendai virus or of other viruses. Examples of publications disclosing oligonucleotide transfer into cells using the liposome technique are, e.g., Meyer et al (1998), Kita et al

(1999), Nakamura et al (1998), Abe et al (1998), Soni et al (1998), Bai et al (1998), see also discussion in the same Journal p. 819-20, Bochot et al (1998), Noguchi et al (1998), Yang et al (1998), Kanamaru et al (1998), and references therein. The use of Lipofectin in liposome-mediated oligonucleotide uptake is described in Sugawa et al (1998). The use of fusogenic cationic-lipid-reconstituted influenza-virus envelopes (cationic virosomes) is described in Waelti et al (1998).

[0185] The above-mentioned cationic or non-ionic lipid agents not only serve to enhance uptake of oligonucleotides into cells, but also improve the stability of oligonucleotides that have been taken up by the cell.

XII. Ribozymes

[0186] Instead of an antisense sequence as discussed herein above, ribozymes can be utilized. This is particularly necessary in cases where antisense therapy is limited by stoichiometric considerations (Sarver et al, 1990). Ribozymes can then be used that will target the same sequence. Ribozymes are RNA molecules that possess RNA catalytic ability (see Cech for review) that cleave a specific site in a target RNA. The number of RNA molecules that are cleaved by a ribozyme is greater than the number predicted by stochiochemistry. (Hampel et al, 1989; Uhlenbeck, 1987).

[0187] Given the known mRNA sequence of a gene, ribozymes, which are RNA molecule that specifically bind and cleave said mRNA sequence (see, e.g., Chen et al (1992), Zhao et al (1993), Shore et al (1993), Joseph et al (1993), Shimayama et al (1993), and Cantor et al (1993), may be designed.

[0188] Ribozymes catalyze the phosphodiester bond cleavage of RNA. Several ribozyme structural families have been identified including Group I introns, RNase P, the hepatitis delta virus ribozyme, hammerhead ribozymes and the hairpin ribozyme originally derived from the negative strand of the tobacco ringspot virus satellite RNA (sTRSV) (Sullivan, 1994; U.S. Patent NO 5,225,347, columns 4-5). The latter two families are derived from viroids and virusoids, in which the ribozyme is believed to separate monomers from oligomers created during rolling circle replication (Symons, 1989 and 1992). Hammerhead and hairpin ribozyme motifs are most commonly adapted for trans-cleavage of mRNAs for gene therapy (Sullivan, 1994). The ribozyme type utilized in the present invention is selected as is known in the art. Hairpin ribozymes are now in clinical trial and are the preferred type. In general the ribozyme is from 30-100 nucleotides in length.

[0189] Accordingly, a ribozyme-encoding RNA sequence may be designed that cleaves the mRNA of a bad gene of the present

invention. The site of cleavage is preferably located in the coding region or in the 5' non-translated region, more preferably, in the 5' part of the coding region close to the AUG translational start codon.

[0190] A DNA encoding a ribozyme according to the present invention may be introduced into cells by way of DNA uptake, uptake of modified DNA (see modifications for oligonucleotides and proteins that result in enhanced membrane permeability, as described above for oligonucleotides and described below for proteins), or viral vector-mediated gene transfer.

XIII. Negative Dominant Peptides

[0191] Negative dominant peptide refers to a peptide encoded by a cDNA sequence that encodes only a part of a protein, i.e. a peptide (see Herskowitz, 1987). This peptide can have a different function from the protein it was derived from. It can interact with the full protein and inhibit its activity or it can interact with other proteins and inhibit their activity in response to the full protein. Negative dominant means that the peptide is able to overcome the natural proteins and fully inhibit their activity to give the cell a different characteristic, such as resistance or sensitization to killing. For therapeutic intervention either the peptide itself is delivered as the active ingredient of a

pharmaceutical composition or the cDNA can be delivered to the cell utilizing the same methods as for antisense delivery.

XIV. Introduction of Proteins, Peptides, and DNA into Cells

[0192] The present invention provides proteins encoded by good genes, peptides derived therefrom, antisense DNA molecules corresponding to bad genes, peptides which are negative dominant for bad genes, and oligonucleotides. A therapeutic or research-associated use of these tools necessitates their introduction into cells of a living organism or into cultured cells. For this purpose, it is desired to improve membrane permeability of peptides, proteins and oligonucleotides. Ways to improve membrane permeability of oligonucleotides have been discussed above. The same principle, namely, derivatization with lipophilic structures, may also be used in creating peptides and proteins with enhanced membrane permeability. For instance, the sequence of a known membranotropic peptide as noted above may be added to the sequence of the peptide or protein. Further, the peptide or protein may be derivatized by partly lipophilic structures such as the above-noted hydrocarbon chains, which are substituted with at least one polar or charged group. For example, lauroyl derivatives of peptides have been described by Muranishi et al (1991). Further modifications of peptides and proteins include the oxidation of methionine residues to

thereby create sulfoxide groups, as described by Zacharia et al (1991). Zacharia and coworkers also described peptide or derivatives wherein the relatively hydrophobic peptide bond is replaced by its ketomethylene isoester ($COCH_2$). It is known to those of skill in the art of protein and peptide chemistry these and other modifications enhance membrane permeability.

[0193] Another way of enhancing membrane permeability is to make use of receptors, such as virus receptors, on cell surfaces in order to induce cellular uptake of the peptide or protein. This mechanism is used frequently by viruses, which bind specifically to certain cell surface molecules. Upon binding, the cell takes the virus up into its interior. The cell surface molecule is called a virus receptor. For instance, the integrin molecules CAR and AdV have been described as virus receptors for Adenovirus (Hemmi et al, 1998, and references cited therein). The CD4, GPR1, GPR15, and STRL33 molecules have been identified as receptors/ coreceptors for HIV (Edinger et al, 1998 and references cited therein).

[0194] By conjugating peptides, proteins or oligonucleotides to molecules that are known to bind to cell surface receptors, the membrane permeability of said peptides, proteins or oligonucleotides will be enhanced. Examples of suitable groups for forming conjugates are sugars, vitamins,

hormones, cytokines, transferrin, asialoglycoprotein, and the like molecules. Low et al U.S. Patent 5,108,921 describes the use of these molecules for the purpose of enhancing membrane permeability of peptides, proteins and oligonucleotides, and the preparation of said conjugates.

[0195] Low and coworkers further teach that molecules such as folate or biotin may be used to target the conjugate to a multitude of cells in an organism, because of the abundant and non-specific expression of the receptors for these molecules.

[0196] The above use of cell surface proteins for enhancing membrane permeability of a peptide, protein or oligonucleotide of the invention may also be used in targeting the peptide, protein or oligonucleotide of the present invention to certain cell types or tissues. For instance, if it is desired to target neural cells, it is preferable to use a cell surface protein that is expressed more abundantly on the surface of those cells.

[0197] The protein, peptide or oligonucleotide of the invention may therefore, using the above-described conjugation techniques, be targeted to a certain cell type. For instance, if it is desired to protect from neurotoxic stress in neural cell, a good gene, or protein encoded thereby, or an antisense or ribozyme of the invention designed to inhibit a bad gene, may be targeted at such cells, for instance, by using

molecules that are expressed on these cells. The skilled person will recognize the possibilities of using a cell surface marker selected from a multitude of known markers of neural and other cells, and of these, further selecting those that are expressed constitutively or inducibly.

XV. Virus-Mediated Cellular Targeting

[0198] The proteins, peptides and antisense sequences of the present invention may be introduced into cells by the use of a viral vector. The use of a vaccinia vector for this purpose is described in Chapter 16 of Ausubel et al (1994-2000). The use of adenovirus vectors has been described, e.g., by Teoh et al (1998), Narumi et al (1998), Pederson et al (1998), Guang-Lin et al (1998), and references therein, Nishida et al (1998), Schwarzenberger et al (1998), and Cao et al (1998). Retroviral transfer of antisense sequences has been described by Daniel et al (1998). The use of SV-40 derived viral vectors and SV-40 based packaging systems has been described by Fang et al (1997). The use of papovaviruses which specifically target B-lymphocytes, has been described by Langner et al (1998).

[0199] When using viruses as vectors, the viral surface proteins are generally used to target the virus. As many viruses, such as the above adenovirus, are rather unspecific in their cellular tropism, it may be desirable to impart

further specificity by using a cell-type or tissue-specific promoter. Griscelli et al (1998) teach the use of the ventricle-specific cardiac myosin light chain 2 promoter for heart-specific targeting of a gene whose transfer is mediated by adenovirus.

[0200] Alternatively, the viral vector may be engineered to express an additional protein on its surface, or the surface protein of the viral vector may be changed to incorporate a desired peptide sequence. The viral vector may thus be engineered to express one or more additional epitopes which may be used to target said viral vector. For instance, cytokine epitopes, MHC class II-binding peptides, or epitopes derived from homing molecules may be used to target the viral vector in accordance with the teaching of the invention. The above Langer et al. (1998) reference teach the use of heterologous binding motifs to target B-lymphotrophic papoaviruses.

XVI. Pharmaceutical Compositions

[0201] The pharmaceutical compositions of the invention are prepared generally as known in the art. Thus, pharmaceutical compositions comprising nucleic acids, e.g., ribozymes, antisense RNA or antisense oligonucleotides, are prepared as described above for pharmaceutical compositions comprising oligonucleotides and/or antisense RNA. The above

considerations apply generally also to other pharmaceutical compositions. For instance, the pharmaceutical composition of the invention may contain naked DNA, e.g., good genes or fragments or derivatives thereof and a pharmaceutically acceptable carrier as known in the art. A variety of ways to enhance uptake of naked DNA is known in the art. For instance, cationic liposomes (Yotsuyanagi et al, 1998), dicationic amphiphiles (Weissig et al, 1998), fusogenic liposomes (Mizuguchi et al, 1996), mixtures of stearyl-poly(L-lysine) and low density lipoprotein (LDL), (terplex, Kim et al, 1998), and even whole bacteria of an attenuated mutant strain of *Salmonella typhimurium* (Paglia et al, 1998) have been used in the preparation of pharmaceutical compositions containing DNA.

[0202] Administration of virus particles has been described in prior art publications, see, e.g., U.S. Patent 5,882,877, where Adenovirus based vectors and administration of the DNA thereof is described. The viral DNA was purified on a CsCl gradient and then dialyzed against Tris-buffered saline to remove CsCl. In these preparations, viral titers (pfu/ml) of 10^{14} to 10^{10} are preferably used. Administration of virus particles as a solution in buffered saline, to be preferably administered by subcutaneous injection, is known from U.S. Patent 5,846,546. Croyle and coworkers (Croyle et al, 1998)

describe a process for the preparation of a pharmaceutical composition of recombinant adenoviral vectors for oral gene delivery, using CsCl gradients and lyophilization in a sucrose-containing buffer.

[0203] The active ingredients of the pharmaceutical composition can include oligonucleotides that are nuclease resistant needed for the practice of the invention or a fragment thereof shown to have the same effect targeted against the appropriate sequence(s) and/or ribozymes. Combinations of active ingredients as disclosed in the present invention can be used including combinations of antisense sequences.

[0204] Where the pharmaceutical composition of the invention includes a peptide or protein according to the present invention, the composition will generally contain salts, preferably in physiological concentration, such as PBS (phosphate-buffered saline), or sodium chloride (0.9% w/v), and a buffering agent, such as phosphate buffer in water or in the well-known PBS buffer. In the following section, the term "peptide" is meant to include all proteins or peptides according to the invention. The preparation of pharmaceutical compositions is well known in the art, see e.g., U.S. Patents 5,736,519, 5,733,877, 5,554,378, 5,439,688, 5,418,219,

5,354,900, 5,298,246, 5,164,372, 4,900,549, 4,755,383, 4,639,435, 4,457,917, and 4,064,236.

[0205] The peptide of the present invention, or a pharmacologically acceptable salt thereof is preferably mixed with an excipient, carrier, diluent, and optionally, a preservative or the like, pharmacologically acceptable vehicles as known in the art, see, e.g., the above U.S. patents. Examples of excipients include, glucose, mannitol, inositol, sucrose, lactose, fructose, starch, corn starch, microcrystalline cellulose, hydroxypropylcellulose, hydroxypropyl-methylcellulose, polyvinylpyrrolidone and the like. Optionally, a thickener may be added, such as a natural gum, a cellulose derivative, an acrylic or vinyl polymer, or the like.

[0206] The pharmaceutical composition is provided in solid, liquid or semi-solid form. A solid preparation may be prepared by blending the above components to provide a powdery composition. Alternatively, the pharmaceutical composition is provided as a lyophilized preparation. The liquid preparation is provided preferably as an aqueous solution, aqueous suspension, oil suspension or microcapsule composition. A semi-solid composition is provided preferably as hydrous or oily gel or ointment. About 0.001 to 60 w/v %, preferably

about 0.05 to 25 w/v % of peptide is provided in the composition.

[0207] A solid composition may be prepared by mixing an excipient with a solution of the peptide of the invention, gradually adding a small quantity of water, and kneading the mixture. After drying, preferably *in vacuo*, the mixture is pulverized. A liquid composition may be prepared by dissolving, suspending or emulsifying the peptide of the invention in water, a buffer solution or the like. An oil suspension may be prepared by suspending or emulsifying the peptide of the invention or protein in an oleaginous base, such as sesame oil, olive oil, corn oil, soybean oil, cottonseed oil, peanut oil, lanolin, petroleum jelly, paraffin, Isopar, silicone oil, fatty acids of 6 to 30 carbon atoms or the corresponding glycerol or alcohol esters. Buffers include Sorensen buffer (Ergeb Physiol, 12:393, 1912), Clark-Lubs buffer (J Bact, 2 (1):109, 191, 1917), Macllvaine buffer (J Biol Chem, 49:183, 1921), Michaelis buffer (Die Wasserstoffinonenkonzentration, p. 186, 1914), and Kolthoff buffer (Biochem Z, 179:410, 1926).

[0208] A composition may be prepared as a hydrous gel, e.g., for transnasal administration. A hydrous gel base is dissolved or dispersed in aqueous solution containing a

buffer, and the peptide of the invention, and the solution warmed or cooled to give a stable gel.

[0209] Preferably, the peptide of the invention is administered through intravenous, intramuscular or subcutaneous administration. Oral administration is expected to be less effective, because the peptide may be digested before being taken up. Of course, this consideration may apply less to a peptide of the invention which is modified, e.g., by being a cyclic peptide, by containing non-naturally occurring amino acids, such as D-amino acids, or other modifications which enhance the resistance of the peptide to biodegradation. Decomposition in the digestive tract may be lessened by use of certain compositions, for instance, by confining the peptide of the invention in microcapsules such as liposomes. The pharmaceutical composition of the invention may also be administered to other mucous membranes. The pharmaceutical composition is then provided in the form of a suppository, nasal spray or sublingual tablet. The dosage of the peptide of the invention may depend upon the condition to be treated, the patient's age, bodyweight, and the route of administration, and will be determined by the attending physician.

[0210] The uptake of a peptide of the invention may be facilitated by a number of methods. For instance, a non-toxic

derivative of the cholera toxin B subunit, or of the structurally related subunit B of the heal-labile enterotoxin of enterotoxic *Eschericia coli* may be added to the composition, see U.S. Patent 5,554,378.

[0211] In another embodiment, the peptide of the invention is provided in a pharmaceutical composition comprising a biodegradable polymer selected from poly-1,4-butylene succinate, poly-2,3-butylene succinate, poly-1,4-butylene fumarate and poly-2,3-butylene succinate, incorporating the peptide of the invention as the pamoate, tannate, stearate or palmitate thereof. Such compositions are described, e.g., in U.S. Patent 5,439,688.

[0212] In a further embodiment, a composition of the invention is a fat emulsion. The fat emulsion may be prepared by adding to a fat or oil about 0.1-2.4 w/w of emulsifier such as a phospholipid, an emulsifying aid, a stabilizer, mixing mechanically, aided by heating and/or removing solvents, adding water and isotonic agent, and optionally, adjusting adding the pH agent, isotonic agent. The mixture is then homogenized. Preferably, such fat emulsions contain an electric charge adjusting agent, such as acidic phospholipids, fatty acids, bilic acids, and salts therof. Acidic phospholipids include phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic

acid. Bilic acids include deoxycholic acid, and taurocholic acid. The preparation of such pharmaceutical compositions is described in U.S. Patent 5,733,877.

[0213] The pharmaceutical compositions containing the active ingredients of the present invention as described herein above are administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the medical arts. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the medical arts. The pharmaceutical compositions can be combinations of the active ingredients but will include at least one active ingredient.

[0214] The doses can be single doses or multiple doses over a period of several days. The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the patient species being treated.

[0215] In one embodiment, the compound of the present invention can be administered initially by intravenous injection to bring blood levels to a suitable level. The patient's levels are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition and as indicated above, can be used. The quantity to be administered will vary for the patient being treated and will vary from about 100 ng/kg of body weight to 100 mg/kg of body weight per day and preferably will be from 10 μg/kg to 10 mg/kg per day.

XVII. Knock-Out or Transgenic Animals

[0216] **Transgenic Mice.** The introduction of gene constructs into the genome of mice (transgenic mice) is a well-established procedure. Transgenic mice provide the opportunity to examine the phenotypic outcome of over-expression or ectopic expression of genes (gain-of-function experiments). Specific phenotypes obtained after such expression is a very strong predictor of gene function. Many human genes have been expressed in transgenic mice and in most cases they function appropriately. Thus ,for the purpose of examining gain-of-function, human genes can be used. Specific plasmid vector constructs are available. They carry any of a variety of promoters that allow expression of the gene in specific tissues. For example, promoters that are brain

specific are available, liver specific promoters, vascular-endothelial cell specific promoters, bone specific promoters, cardiac muscle specific promoters and many more. While mice are specifically discussed herein as the transgenic animal, those of ordinary skill in the art well understand that any other eukaryotic animal can be used in the same way as described for mice to make a corresponding transgenic animal.

[0217] **Knockout Mice.** Loss-of-function experiments in mice are mostly done by the technique of gene knockout. The technology is well established. It requires the use of mouse genes for the purpose of generating knockout of the specific gene in embryonic stem (ES) cells that are then incorporated into the mouse germ-line cells from which mice carrying the gene knockout are generated. From a human gene there are several ways to recover the homologous mouse gene. One way is to use the human gene to probe mouse genomic libraries of lambda phages, cosmids or BACs. Positive clones are examined and sequenced to verify the identity of the mouse gene. Another way is to mine the mouse EST database to find the matching mouse sequences. This can be the basis for generating primer-pairs or specific mouse probes that allow an efficient screen of the mouse genomic libraries mentioned above by PCR or by hybridization. For the vast majority of genes the mouse homologue of the human gene retains the same

biological function. The loss-of-function experiments in mice indicate the consequences of absence of expression of the gene on the phenotype of the mouse and the information obtained is applicable to the function of the gene in humans. On many occasions a specific phenotype observed in knockout mice was similar to a specific human inherited disease and the gene was then proved to be involved and mutated in the human disease. While mice are specifically discussed herein as the knockout animal, those of ordinary skill in the art well understand that any other eukaryotic animal can be used in the same way as described for mice to make a corresponding knockout animal.

[0218] The transgenics and knock-outs of the present invention are constructed using standard methods known in the art and as set forth in United States Patents 5,487,992, 5,464,764, 5,387,742, 5,360,735, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,385, 5,175,384, 5,175,383, 4,736,866 as well as Burke et al (1991), Capecchi (1989), Davies et al (1992), Dickinson et al (1993), Duff et al (1995), Huxley et al (1991), Jakobovits et al (1993), Lamb et al (1993), Pearson et al (1993), Rothstein (1991), Schedl et al (1993), Strauss et al (1993). Further, patent applications WO 94/23049, WO 93/14200, WO 94/06908, WO 94/28123 also provide information.

[0219] More specifically, any techniques known in the art can be used to introduce the transgene expressibly into animals to produce the parental lines of animals. Such techniques include, but are not limited to, pronuclear microinjection (U.S. patent 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al, 1985); gene targeting in embryonic stem cells (Thompson et al, 1989; Mansour, 1990 and U.S. patent 5,614,396); electroporation of embryos (Lo, 1983); and sperm-mediated gene transfer (Lavitrano et al, 1989). For a review of such techniques see Gordon (1989).

[0220] Further, one parent strain instead of carrying a direct human transgene can have the homologous endogenous gene modified by gene targeting such that it approximates the transgene. That is, the endogenous gene has been "humanized" and/or mutated (Reaume et al, 1996). It should be noted that if the animal and human sequence are essentially homologous a "humanized" gene is not required. The transgenic parent can also carry an over expressed sequence, either the non-mutant or a mutant sequence and humanized or not as required. The term transgene is therefore used to refer to all these possibilities.

[0221] Additionally, cells can be isolated from the offspring which carry a transgene from each transgenic parent

and that are used to establish primary cell cultures or cell lines as is known in the art.

[0222] Where appropriate, a parent strain will be homozygous for the transgene. Additionally, where appropriate, the endogenous non-transgene in the genome that is homologous to the transgene will be non-expressive. By non-expressive is meant that the endogenous gene will not be expressed and that this non-expression is heritable in the offspring. For example, the endogenous homologous gene could be "knocked-out" by methods known in the art. Alternatively, the parental strain that receives one of the transgenes could carry a mutation at the endogenous homologous gene rendering it non-expressed.

XVIII. Promoters

[0223] As promoters and regulatory elements of the candidate genes in accordance with the present invention are also useful in the screening assays described in Section VIII, the present invention is also directed to the sequence of such promoters and/or other regulatory agents. Once the gene has been identified, it is within the routine skill in the art for one ordinary skill to identify the sequence of the promoter region or other regulatory regions. This may be accomplished as discussed below.

[0224] It is well recognized that promoters are generally located upstream of the coding sequence. There are numerous methods used conventionally in the art for determining a promoter region and portions of that region essential for promoter activity. For example, Kähäri et al (1990) made constructs in which a region from -2260 to -14 upstream of the ATG initiation codon of the human elastin gene was systematically truncated from -2260 towards -14 to create a set of nested deletions, all with the same -14 end point, which is linked to and controls the expression of a coding sequence for a reporter molecule (chloramphenicol acetyltransferase). The constructs are assayed for the expression of the reporter as a measure of the promoter activity of the truncated DNA fragments. Using this type of deletion analysis, Kähäri et al isolated a 497 bp fragment which provided maximal gene expression.

[0225] The above method is directed to locating the promoter region, as well as identifying the portions thereof essential for activity. Other mutagenesis techniques, such as linker scanning, which generate a series of clustered point mutations can also be used to fine map the sequence elements required for promoter function.

[0226] Although in a great majority of cases the 5'-flanking region is sufficient to promote gene expression, it

has been reported that in some instances intron, or even the 3'-untranslated sequences, provide regulatory sequences that contribute to promoter activity. For example, intron I sequences were found to be important for high-level and tissue-specific expression of an alpha-skeletal actin gene, a beta-globin gene and a peripherin gene (Reecy et al, 1998; James-Pederson et al, 1995; Belecky-Adams et al, 1993). In view of these examples of introns or 3'-untranslated sequences contributing to promoter activity, promoter constructs (i.e., fused to reporter gene) may include intron I sequences of the candidate gene and, when necessary, 3'-untranslated sequences. In the former case, a DNA fragment can be isolated that spans the 5'-flanking region, the first exon and the first intron, followed by the reporter gene. The translation initiation codon of the candidate gene could also be mutated to avoid translation of truncated candidate gene product.

XIX. Examples

General Methods

[0227] Most of the techniques used in molecular biology are widely practiced in the art, and most practitioners are familiar with the standard resource materials which describe specific conditions and procedures. However, for convenience, the following paragraphs can serve as a guideline.

[0228] General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al (1989), and in Ausubel et al (1989), particularly for the Northern Analysis and *in situ* analysis and in Perbal (1988), and in Watson et al. Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990).

[0229] Reactions and manipulations involving other nucleic acid techniques, unless stated otherwise, were performed as generally described in Sambrook et al (1989), and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference.

[0230] Additionally, *in situ* (In cell) PCR in combination with flow cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al, 1996).

[0231] General methods in immunology: Standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al (1994) and Mishell et al (1980). Available immunoassays are extensively described in the patent and scientific literature. See, for example,

United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as Sambrook et al (1989).

General Methods of the Invention

[0232] The general methods of the invention are generally as described in US patent application serial number USSN 09/309,862 of same applicant which is by reference incorporated herein in its entirety.

[0233] In brief, USSN 09/309,862 provides methods for identifying genes regulated at the RNA level by cue-induced gene expression. It relates to the rapid isolation of differentially expressed or developmentally regulated gene sequences through analysis of mRNAs obtained from specific cellular compartments and comparing the changes in the relative abundance of the mRNA in these compartments as a result of applying a cue to the tested biological samples. The cellular compartments include polysomal and non-polysomal fractions, nuclear fractions, cytoplasmic fractions and splicesomal fractions. The method includes the steps of exposing cells or tissue to a cue or stimulus such as mechanical, chemical, toxic, pharmaceutical or other stress, hormones, physiological disorders or disease; fractionating

the cells into compartments such as polysomes, nuclei, cytoplasm and splicesomes; extracting the mRNA from these fractions, and subjecting the mRNA to differential analysis using accepted methodologies, such as gene expression array (GEM).

[0234] The method is designed for identifying and cloning genes which are either up- or down-regulated responsive to a specific pathology, stress, physiological condition, and so on, and in general, to any factor that can influence cells or organisms to alter their gene expression.

[0235] Further in USSN 09/309,862, an example is provided which shows the use of RNA isolation from nuclei for isolating genes whose steady state levels show only minor changes, but which show high differential expression when detected by nuclear RNA probe. Most such genes are regulated at the transcriptional level.

[0236] The specific mRNA of the invention is total cellular mRNA, and regulation is specifically on the transcriptional level.

[0237] In order to identify genes whose expression is either induced or reduced by hypoxia, the following experimental techniques were conducted.

Preparation of Custom Hypoxia-Specific Microarrays

[0238] The first step in identifying the genes of the present invention involves the preparation of a microarray containing genes which are suspected of either being induced by hypoxia after 16 hours, reduced by hypoxia after 16 hours, or induced by hypoxia after 4 hours, which genes are obtained either from the rat C6 glioma cell line or the human A172 glioma cell line.

[0239] In the preparation of such a microarray, each of the cell lines were exposed to hypoxic conditions (0.5% O_2 and 5% CO_2) for 4 or 16 hours and compared to cells grown under normal conditions (normoxia). Three enriched libraries were made by the suppression subtractive hybridization (SSH) method using the "PCR-Select cDNA subtraction kit" from CLONTECH. The subtractive libraries were made from the following sample:

1. 16 hours hypoxia vs. normal (genes induced by hypoxia after 16 hours).

2. normal vs. 16 hours hypoxia (genes reduced by hypoxia after 16 hours).

3. 4 hours hypoxia vs. normal (genes induced by hypoxia after 4 hours).

[0240] From library 1, 1000 colonies were grown, and the plasmids prepared in 96 well format. From libraries 2 and 3, 500 colonies were processed from each. Thus, a total of 2000

individual plasmids were prepared and used for the fabrication of a Gene Expression Microarray (GEM). For this, the inserts of each plasmid were amplified by PCR and robotically fabricated on the glass. cDNA chip printing was performed by Synteni (Wang et al, 1999)

<u>Preparation of Probes for Microarray Hybridization</u>

[0241] Isolated messenger RNA is labeled with fluorescent dNTP's using a reverse transcription reaction, using 50 µg template RNA (probes derived from nuclear RNA and total RNA), to generate a labeled cDNA probe. mRNA is extracted from either C6 or A172 cells cultured in normoxia conditions and labeled with Cy3-dCTP (Amersham) and mRNA extracted from C6 or A172 cells cultured under hypoxic conditions is labeled with Cy5-dCTP (Amersham). The two labeled cDNA probes are then mixed and hybridized onto microarrays (Schena et al, 1996; Wang et al, 1999). Following hybridization the microarray was scanned using a laser scanner and the amount of fluorescence of each of the fluorescence dyes was measured for each cDNA clone on the microarray giving an indication of the level of mRNA in each of the original mRNA populations being tested. Comparison of the fluorescence on each cDNA clone on the microarray between the two different fluorescent dyes is a measure for the differential expression of the indicated genes between the two experimental conditions.

[0242] The following probes were made from C6 and A172 for screening the GEM:

1. Normoxia (Cy3 labeled) + 16 hours hypoxia (Cy5 labeled).
2. Normoxia (Cy3 labeled) + 4 hours hypoxia (Cy5 labeled).

[0243] The following cDNA sequences of the present invention were found to be induced under hypoxic conditions.

In Situ Analysis:

[0244] *In situ* analysis is performed for the candidate genes identified by the differential response to exposure to hypoxic conditions as described above. The expression is studied in normal tissues and in pathological models as described herein.

[0245] Utilizing microarray hybridization the sequences set forth herein were identified and cloned as being differentially expressed under hypoxic conditions (see also Braren et al, 1997).

[0246] In parallel experiments Northern Analysis results and results obtained by the gene expression microarray analysis where found to coincide and either can be used to determine hypoxia-regulated response. As well in other experiments, the results from *in situ* analysis showed a high

degree of correlation with the Northern Analysis and microarray analysis.

[0247] The sequences are listed that were found, the sequences are identified by clone number. In some cases either end of the clone has been sequenced for use or the entire clone sequence and protein sequence are provided.

[0248] Unigem1 (Syntheni) was utilized for screening of human glioma cell line A172 to identify genes whose expression is modified by hypoxia.

<u>A Retinopathy Model:</u>

[0249] Three major biological processes occur in nervous tissues under hypoxic conditions:

1. <u>apoptotic death</u> of hypoxia-damaged cells;
2. <u>angiogenesis</u> induced by factors secreted by hypoxia-suffering cells (a feedback control of oxygen concentration in tissue); and
3. secretion of neurotrophic and neuroprotective factors.

[0250] Therefore, it was assumed that among novel genes transcriptionally regulated by hypoxia in C6 and A172 glioma cells, there are those with pro- and antiapoptotic function as well as secreted neurotrophic, neuroprotective and angiogenic factors. It is worth noting, that regulation of apoptosis and angiogenesis is closely linked to cancerogenesis.

[0251] As initial step of biological characterization, candidate genes were tested for their ability to induce/protect cells from apoptosis, for neurotrophic activity and for angiogenic/antiangiogenic activity.

Cell Culture

[0252] MCF7 Tet-off (Clontech) human epithelial breast carcinoma cells and their transfected derivatives were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 20 U/ml penicillin, 20 μg/ml streptomycin, and 100 μg/ml neomycin. The transfectants were cultured in the presence of 1 μg of tetracycline per ml. For UV treatment, cells were irradiated with 100 Mj/cm^2 short wavelength UV (UV Crosslinker, Fisher) and then incubated at 37°C for 24 hours. Cells were stained with 0.5% methylene blue in 50% ethanol.

[0253] Human umbilical vein endothelial cells (HUVEC) were grown in M199 medium supplemented with 20% FCS, 2 mM L-glutamine, 20 U/ml penicillin, 20 μg/ml streptomycin, 0.001 mg/ml Heparin, 0.1 mg/ml ECGS.

Expression Vectors and Transfection Methods

[0254] pTet-Splice/95 flag construct was prepared by EcoRI/HindIII subcloning from pLPC flag into pTet-splice.

[0255] MCF7 Tet-off cells were transfected with pTet-Splice/95 Flag using lipofectamine reagent. Stable transfectants were obtained by cotranfection of 0.5 μg of a

thymidine kinase hygromycin plasmid. Cells were selected with 100 µg per ml hygromycin in the presence of 2 µg per ml tetracycline in the medium. Clones were screened for tetracycline-sensitive HP95 expression by Northern blot.

Growth Rate Analysis

[0256] MCF7 cells and their HP95 transfectants were seeded at 10^4 cells per 35-mm-diameter dish with or without tetracycline. At daily intervals, cells were collected by trypsinization and counted. This experiment was done in triplicate.

Assessment of Cell Viability

[0257] The cell viability was estimated by the lactate dehydrogenase (LDH) leakage method using a Cytotoxicity Detection Kit (Molecular Biochemicals) according to the manufacturer's protocol. LDH activity was measured as the optimal density at 492 nm.

Ischemia

[0258] Ischemia was achieved by incubating cells in a glucose free medium in a humidified environment at 37^0C in a three gas incubator maintained at 5% CO_2 and 0.5% O_2 for 16 hours.

Oxidative Stress

[0259] MCF7 cells were treated by adding to complete medium freshly prepared hydrogen peroxide at the concentration of 1mM for 24 hours.

Serum starvation Experiment

[0260] MCF7 clones were plated at 10^4 cells in six-well plates in DMEM containing 10% FCS with or without tetracycline. The medium was replaced 72 hours later with medium containing 0.1% serum in the presence or absence of tetracycline. After 24 hours cell viability was measured.

Annexin V Apoptosis Assay

[0261] The MCF7 clones were seeded into 60 mm culture dishes (1×10^5 cells/dish) and were maintained in the presence or absence of tetracycline for 72 hours. The cells were collected by trypsinization, centrifuged and washed in phosphate-buffered saline (PBS). The cells were then resuspended in 200 µl of 1X binding buffer. The apoptotic cells were analyzed using a Annexin V apoptosis assay kit (ALEXIS Biochemicals) according to the manufacturer's protocol.

Western Blot Analysis

[0262] Cells were washed with phosphate-buffered saline, and lysed in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 1% (v/v) Noidet P40, 0.1% (w/v) sodium deoxycholate, 0.1%

(w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15 M NaCl, and protease inhibitor cocktail (Boehringer Mannheim). The whole cell lysates were clarified by centrifugation at 12,000 × g for 30 minutes. Lysates containing 30 μg of protein were fractionated by SDS- 10% polyacrylamide gel and transferred onto membrane (Schleicher & Schuell). The blots were incubated with antibody specific for Bcl-2 (Transduction Laboratories) and with the second antibody for detection of Bcl-2 using the ECL detection system (Amersham).

Collection of Conditioned Medium

[0263] The MCF7 clones (1×10^5 cells/dish) were grown in HUVEC medium in the presence or absence of tetracycline for 72 hours. Cell-conditioned media were collected, centrifuged at 15,000 × g for 10 minutes. HUVEC, MCF7 and PC12 cells were seeded into 6 wells culture dishes (3×10^4 cells/well) 72 hours later, the conditioned medium was added (1:1). After 24 hours cell viability was measured.

Middle Cerebral Artery Occlusion (MCAO) Stroke Model

[0264] The stroke model was implied in the stroke-prone spontaneously hypertensive rat strain. Occlusion was permanent and unilateral - by electrocoagulation of MCA. This led to focal brain ischemia at the ipsilateral side of brain cortex leaving the contralateral side intact (control). Experimental animals were sacrificed 1, 2, 4, 12, 24, 48 and

72 hours after the operation, respectively. Brains were removed, fixed in formalin, embedded into paraffin and coronal sections were performed for the further use in *in situ* hybridization with Hypoptin-specific riboprobes. VEGF- and PGK-specific riboprobes were used as positive controls.

In Situ Hybridization

[0265] Radioactive *in situ* hybridization was performed according to previously published protocol (Faerman et al, 1997) with slight modifications. Deparaffinized sections were heated in 2XSSC at 70°C for 30 minutes, rinsed in distilled water and incubated with 10 mg/ml proteinase K in 0.2M Tris-HCl (pH7.4), 0.05 M EDTA at 37°C for 20 minutes. After proteinase digestion, slides were postfixed in 4% paraformaldehyde in PBS (20 minutes), quenched in 0.2% glycine (5 minutes), rinsed in distilled water, rapidly dehydrated through graded ethanols and air-dried. The hybridization mixture contained 50% formamide, 4xSSC (pH 8.0), 1XDenhardt's, 0.5 mg/ml herring sperm DNA, 0.25 mg/ml yeast RNA, 10 mM DTT, 10% dextran sulfate and 2×10^4 cpm/μl of [^{35}S]-UTP-labeled riboprobe. After application of the hybridization mixture, sections were covered with sheets of polypropylene film cut from autoclavable disposposal bags and incubated in humidified chamber at 65°C overnight. After hybridization covering film was floated off in 5xSSC with 10 mM DTT at 65°C and slides were

washed at high stringency: 2XSSC, 50% formamide, 10 mM DTT at 65°C for 30 minutes and treated with RNAse A (10 µg/ml) for 30 minutes at 37°C. The high stringency washing step was repeated and slides were next washed in 2XSSC and 0.1XSSC (15 minutes each) at 37°C. Then slides were rapidly dehydrated through ascending ethanols and air-dried. For autoradiography slides were dipped in Kodak NTB-2 nuclear track emulsion diluted 1:1 with double-distilled water and were exposed for 3 weeks in light-tight box containing dessicant at 4°C. Exposed slides were developed in Kodak D-19 developer, fixed in Kodak fixer and counterstained with hematoxilin-eosin.

[0266] Microphotographs were taken using Zeiss Axioscop-2 microscope equipped with Diagnostic Instruments Spot RT CCD camera.

[0267] The sequences of the invention, the methods used therewith and the utility of the present invention can be shown by the following non-limiting examples:

EXAMPLE 1: 92 (SEQ ID NO:1)

Northern Blot Analysis:

[0268] Gene 92 is found up-regulated after 16 hours of hypoxia. On Northern blots, it appears as a single 5 Kb transcript.

Cloning:

[0269] Several partial human cDNA clones corresponding to gene 92 were isolated from human A172 cDNA library. The length of available contig is 2212 bp and it contains an ORF potentially coding for a 437 amino acid (265-1576 bp) protein (SEQ ID NO:2). The putative initiating ATG codon is preceded by in frame stop codon.

Bioinformatic Analysis:

[0270] Similarity search with 92 cDNA sequence against the public databases have shown 60% similarity to unknown Drosophila DNA sequence (AC004283) and mainly encompasses the 3′ UTR and a part of the coding sequence. The search against the protein public databases gave partial similarity to hypothetical C. elegans protein (1703624) (77% similarity and 46% Identity).

[0271] The 92 cDNA sequence contains a region of 55 nucleotides (336-390 bp) that is constituted of CGG repeats. On the level of amino acids it appears as a GGD/SFGG (SEQ ID NO:20) repeated unit (aa 24-44). Two of the isolated cDNA clones contain a 30 nucleotides in frame deletion within this region, indicating that the amount of repeats can be variable. Forty-four of these nucleotides form a strong stem and loop secondary structure. When 92 cDNA was *in vitro* translated, the obtained protein had much smaller size than expected (30

kD instead of 45 kD). This, means that the stem and loop structure formed right downstream to the putative initiation codon prevents the proper progression of ribosome and the initiation actually starts from the next in frame ATG located at position 820-822.

EXAMPLE 2: 95 (SEQ ID NO:3)

Identification of Gene 95 mRNA Induction under Hypoxic Treatments

[0272] cDNA microarray differential expression was performed in order to identify genes that were responsive to hypoxia in human A172 glioma cells. 95 mRNA levels were significantly elevated under hypoxia. Northern blot analysis was performed in order to verify these observations. The 95 mRNA levels (3.9 kb) were highly induced by hypoxia in A172 glioma cells. Human EST that contained a full-length cDNA was identified as the human 95 transcript (SEQ ID NO:3). By *in vitro* translation this cDNA gave rise to a protein product of 62 Kd (SEQ ID NO:4). The sequence is 480 aa corresponding to nucleotides 323-1762 of SEQ ID NO:3.

[0273] Gene 95 shares homology with the PA26 gene (Fig. 1). However, PA26 mRNA levels were not influenced by hypoxia in A172 cells (results not shown). Incubation of various cell lines from different origin (H1299, MCF7, Skov3) revealed high induction of 95 mRNA after 4 and 16 hours of hypoxic

treatments (results not shown). p53 was not essential for the hypoxia-induced up-regulation, since 95 mRNA levels were increased during hypoxia, regardless of the p53 status of the cells.

[0274] The results from testing on a variety of cell lines prove that the hypoxia-induced up-regulation of expression of this gene is not limited to a specific cell line, but is found in a variety of cell lines. This confirms the expectation that such up-regulation will be found in any human cell subjected to hypoxia. Therefore, gene 95 and its encoded protein are excellent candidates for diagnostic testing of tissue or fluids for having been subjected to hypoxia, as described above.

[0275] It is known that HIF-1 mediated gene transactivation involves distinct nucleic acid motifs, namely HREs, in its binding. Accordingly, computer software from Genomatix GmbH was used to analyze the human 95 gene for appropriate HRE sequences. In this analysis, the HRE consensus motif proposed by Wenger et al (1997) was followed. Two putative HRE sites were found in intron 1 and intron 2 of 95 gene (not shown), suggesting an HIF-1-dependent regulation of 95 gene transcription.

Gene 95 mRNA Was Up-Regulated Following DNA Damage in a p53 Dependent Manner

[0276] The effect of DNA damage on 95 mRNA was examined. Different cell lines were exposed to doxorubicin, a DNA-damaging agent that induces DNA breaks, or to UV radiation. 95 mRNA was strongly induced 24 hours after doxorubicin treatment in p53 wild type cells (MCF7, HEF and 293). In contrast, no induction was detected in p53-deficient cells (MDAH041, H1299). Similar results were obtained for cells exposed to UV radiation (data not shown). To verify the hypothesis on regulation of 95 by p53 under DNA-damage, MCF7 and their derivatives transduced with GSE56 (p53 dominant negative) were exposed to doxorubicin, UV radiation and hypoxia. GSE56 completely abrogated the induction of 95 by DNA-damaging agents, but did not affect its induction by hypoxia.

Inducible Expression of 95 in MCF7 Cells Revealed Delay in Their Growth Rate and induced Apoptosis

[0277] To permit conditional expression of a potential antiproliferative gene, human epithelial breast carcinoma MCF7 cells were stably transfected with a tetracycline-repressible vector containing flag-epitope-tagged 95. Two clones of MCF7 cells, which showed tetracycline-sensitive expression of 95 were obtained by Northern blot. To investigate how 95

overexpression affects the growth rate of proliferating breast tumor cells, the growth of the transfectant clones and control clones in the presence or absence of tetracycline was determined. As shown in Fig. 2, 95 overexpressing clones showed significant delay in growth compared with non-induced cells.

[0278] In order to determine whether this growth inhibition was due to 95-induced cell apoptosis, the 95 inducible clones were grown for 72 hours in the presence or absence of tetracycline. Overexpression of 95 resulted in cell apoptosis as determined by Annexin V apoptosis assay. Since it is known that Bcl-2 has a protective effect against apoptosis, its expression in MCF7-95 induced clones by Western analysis was tested. Dephosphorylated-Bcl-2 expression was induced in 95 overexpressing clones.

<u>95 Induced DNA Damaged Apoptosis in MCF7 Cells</u>

[0279] To find whether DNA damaged agents can stimulate apoptosis in 95 overexpressing cells, MCF7-95 inducible clones were treated with doxorubicin or exposed to UV irradiation in the presence or absence of tetracycline. Both stimuli induced apoptosis in >90% of the MCF7-95 expressing cells. Treatment with taxol, which is an antimicrotubule agent, revealed no difference between MCF7-95 inducible and control clones.

[0280] To investigate how 95 overexpression affects the response of proliferating breast tumor cells to mitogens, the response to serum-starved conditions (0.1% serum) was determined. Over-expression of 95 in MCF7 induced serum deprivation cell death, as was assessed by measuring lactate dehydrogenase (LDH) activity released from cells, by a spectrophometric method (Fig. 3).

Conditioned Medium from MCF7-95 Inducible Clones Promoted Cell Death

[0281] In order to determine whether MCF7-95 conditioned medium can stimulate apoptosis in other cells, conditioned medium was collected from MCF7-95 inducible clones that were grown in the presence or absence of tetracycline, and was added to human umbilical vein endothelial cells (HUVEC). After incubation of 24 hours, HUVEC cell death was measured. Conditioned medium from MCF7 clones overexpressing 95 promoted HUVEC cell death. The same phenomena was observed by adding the MCF7-95 conditioned medium to non-transfected MCF7 and PC12 cells.

95 Overexpression Protected MCF7 cells Against Hypoxia and H_2O_2-Induced Cell Death

[0282] To find the roles of 95 in hypoxia-induced cell death, the inducible clones were grown under ischemic conditions in the presence or absence of tetracycline. 95

overexpression protected MCF7 cells against hypoxia-induced cell death, as was assessed by measuring lactate dehydrogenase (LDH) activity released from cells (Fig.4).

[0283] H_2O_2 is a natural product of metabolism, but at sufficient concentrations it produces cell damage. To demonstrate whether H_2O_2 induces apoptosis in MCF7-95 inducible clones, the cells were treated with 1 mM H_2O_2 for 24 hours. As shown in Fig. 5, 95 overexpression protected MCF7 cells against H_2O_2 induced apoptosis.

<u>95 Expression Was Up-regulated in the Brain of a Rat Model of Stroke</u>

[0284] The ^{35}S-labeled probe specific to the gene 95 was hybridized to coronal section of rat brains fixed at different time points (30 minutes, 1 hour, 2 hours, 4 hours, 12 hours, 24 hours, 48 hours, 72 hours) after permanent middle cerebral artery occlusion (MCAO). Results of this *in situ* hybridization study revealed the expression of the gene 95 at 12 and 24 hours after MCAO. Hybridization signal located to the subset of neurons in the transitional zone between the ischemic core and peri-infarct area.

<u>95 Expression Was Up-regulated in Tumors (Not Necessarily Human)</u>

[0285] Sections of tumors grown from C6 glioma cells in nude mice were hybridized to ^{35}S-labeled riboprobe specific to

the gene 95. Results of *in situ* hybridization demonstrated expression of the 95 gene in tumor cells surrounding necrotic areas. This pattern of expression closely resembles that of the VEGF revealed by hybridization of the corresponding probe to the parallel sections. These results suggest activation of the gene 95 expression in hypoxic areas within growing tumors.

Discussion

[0286] HIF-1 is a major regulator of the adaptation of the cells to hypoxic conditions. As observed with ES cells, this transcription factor is necessary to maintain cell proliferation in hypoxia (Iyer et al, 1997). However, HIF-1 is also involved in apoptosis in ES cells (Carmeliet et al, 1998). In hypoxia, HIF-1α is stabilized and is able to interact with p53. This interaction leads to stabilization and increased cellular p53, which, however, inhibits HIF-1α activity (Blagosklonny et al, 1998; An et al, 1998). Coexpression of HIF-1α and wild-type p53 leads to the inhibition of the HIF-1-induced transactivation (Blagosklonny et al, 1998). The dual role of HIF-1 as a necessary factor in survival to hypoxic stresses, but also as a pro-apoptotic protein, is not clear.

[0287] A novel p53 and HIF-1 target gene, 95 has been isolated and characterized. It shares homology with PA26, a member of the GADD family. 95 is up-regulated and induces DNA

damaged apoptosis in a p53 dependent manner. In contrast, 95 is up-regulated and protects MCF7 cells against ischemia and H_2O_2-induced cell death.

[0288] Hydrogen peroxide (H_2O_2) has been known to activate the mitochondrial permeability transition pore and the release of the mitochondrial protein cytochrome c (Stridh et al, 1998; Sugano et al, 1999). In the cytosol, cytochrome c in combination with Apaf-1 activates caspase-9, which then finally leads to activation of caspase-3 and apoptosis (Hampton et al, 1997). Caspases are evolutionarily conserved executioners of programmed cell death in normal development and are also implicated in a variety of pathological conditions, including cerebral ischemia (Nicholson et al, 1997). A recent study provides *in vitro* and *in vivo* evidence that a family of caspases plays a pivotal role in the hypoxia- and ischemia-induced death of oligodendrocytes (Shibata et al, 2000). The present results suggest that 95 is up-regulated by hypoxia, brain ischemia and H_2O_2, and that it plays a suppressive role in ischemia- and H_2O_2-induced apoptosis. Further investigation will be necessary to determine whether caspases are involved in 95 apoptotic machinery.

[0289] Bcl-2, a 26-kDa membrane-anchored proto-oncoprotein, was the first gene product discovered as an apoptosis suppressor acting in various cells (Reed et al, 1994). After

cerebral ischemia, Bcl-2 is induced in surviving neurons (Clark et al, 1997), suggesting its protective effect on ischemic brain injury. Overexpression of Bcl-2 by gene transfer or in transgenic mice reduces the volume of infarction after cerebral ischemia (Martinou et al, 1994; Lawrence et al, 1996). Two mechanisms can be involved in the pro-survival effect of Bcl-2 against ischemic insults. The first is the anti-apoptotic effect of Bcl-2 and the second is its function as an antioxidant (Hockenbery et al, 1993). Recently, it was shown that ischemic insults dephosphorylated Bcl-2 in a time-dependent fashion without affecting the total amount of protein, and suggested that dephosphorylation of serine 70 is one of the critical factors in decreasing the anti-apoptotic function of Bcl-2 (Itakura et al, 2000). The present results show that overexpression of 95 in MCF7 cells induces dephosphorylated-Bcl-2 expression, and suggest that dephosphorylation of Bcl-2 may be involved in 95-induced apoptosis.

[0290] Several previous studies have implicated GADD153 expression in the mechanism of growth arrest and apoptosis (Barone et al, 1994; Chen et al, 1996). Introduction of GADD153 gene into gastric cancer cells can modulate sensitivity to anticancer agents in association with apoptosis (Kim et al, 1999). Furthermore, loss of GADD 153 gene

expression leads to high genetic instability of oral melanoma cells (Korabiowska et al, 1999). In this study, it was shown that introduction of 95 gene into human epithelial breast carcinoma MCF7 cells can modulate their sensitivity to the anticancer agent doxorubicin. 95 has been mapped to 1p34-35 (HTGS), a part of chromosome 1 frequently deleted in high stage neuroblastoma tumors and sporadic breast tumors (Jogi et al, 2000; Phelan et al, 1996). Future mutations analysis of 95 in neuroblastoma and breast tumor samples will answer whether 95 is likely to be involved in the genesis of these tumors.

[0291] GADD153 and 95 could possess functions analogous to traditional stress-response genes, serving to protect cells from stress-induced damage and/or aiding the recovery of normal cellular functions following stress. One way in which p53 is thought to potentiate genomic stability, and consequently inhibit tumorigenesis is the removal of damaged cells through the triggering of apoptosis via transcriptional induction of genes that encode proapoptotic factors, such as 95. This study suggests that 95 induces DNA damage mediated apoptosis in a p53 dependent manner and protects against oxidative stress mediated apoptosis in a p53 independent mechanism. The identification of key events in the apoptotic pathway that are affected by cellular responses, such as the

expression of 95, could facilitate the identification of targets for the manipulation of this protein, which may have important medical implications.

[0292] Accordingly, it is clear that 95 is a good gene and has all of the utilities discussed herein for good genes. Promotion of apoptosis in DNA damaged cells is also a beneficial property. Thus, administration of the 95 gene product to the site of a hypoxic event will help to ameliorate the undesirable effects of such an event.

EXAMPLE 3: 98 (SEQ ID NO:5)

Northern Blot Analysis:

[0293] Expression of gene 98 is strongly up-regulated by hypoxia already after four hours of exposure. On Northern blots, it appears as a single mRNA species of 4.4. Kb.

Cloning:

[0294] A full-length 98 cDNA was cloned. It is 4138 bp long and contains an single ORF encompassing the nucleotides 204-1445. The putative protein is 414 amino acids long.

Bioinformatic Analysis:

[0295] Search of the public databases revealed that 98 encoded protein is similar to two other human proteins: (1) a putative protein encoded by anonymous human 24945 mRNA sequence (AF131826) and (2) VDUP1 (protein induced in HL-60 cells by dihydroxy vitamin D3 treatment) (S73591). No

significant structural features were found by existing protein analysis tools within the 98 putative protein.

[0296] It was previously demonstrated that treatment with vitamin D3 can induce apoptosis in C6 rat glioma cells (Baudet et al, 1996). Therefore, the relationship between the vitamin D-induced cell killing and 98 gene expression and function in glioma cells was studied.

[0297] The mammalian 98 expression vector was then prepared and its effects studied.

EXAMPLE 4: 60F6 (SEQ ID NO: 6)

Northern Blot Analysis:

[0298] Expression of this gene is moderately up-regulated after 16 hours of hypoxia. On Northern blot, it appears as a single 3.0 Kb species.

Cloning:

[0299] A complete 60F6 human cDNA clone was isolated from A172 cDNA library. The contig is 2675 bp long and contains a single ORF (bp 134-866) able to code for a putative protein of 244 amino acids (SEQ ID NO:7).

Bioinformatic Analysis:

[0300] A similarity search against the public databases revealed that the N-terminal half of 60F6 sequence exactly corresponds to a human cDNA coding for RhoE/Rho8 small GTP-binding protein (P52199, HSRHO8GRN). The identity of gene

60F6 was not determined before, because the small sequenced fragment that was initially possessed, originated from the Rho8 long 3′ UTR. All the sequence information available in public databases did not include the long 3′ UTR of Rho8. Structurally, Rho8 belongs to a family of Ras-related GTPases that regulate the actin cytoskeleton. However, this protein is unique in that it is constitutively active: GTPase deficient and *in vivo* farnesylated (Foster et al, 1996). Therefore, it is intriguing to find that this constitutively active G-protein is regulated on the level of transcription. Hypoxia regulation of Rho8 was not previously described.

EXAMPLE 5: 648 (lysyl hydroxylase 2) (SEQ ID NO:8, 10 and 12)

Northern analysis

[0301] Probe 648 has detected a single 3.8 Kb transcript on Northern blots. Expression was induced in C6 glioma cells already after 4 hours of hypoxia.

Cloning

[0302] After extension of initial cDNA probe by RACE it became evident that identified rat sequence (SEQ ID NO:8), encoding a 758 aa of SEQ ID NO:9, is able to code for protein that represents a rat homologue of human lysyl hydroxylase 2 (PLOD2). The full-length open reading frames were cloned for both human (SEQ ID NO:10) and rat (SEQ ID NO:12) lysyl hydroxylase 2 homologues (by PCR, using primers built on the

basis of known sequence, for human variant, and degenerative primers, for rat variant). The encoded proteins (SEQ ID NOs:11 and 13, respectively) have well defined signal peptides.

Bioinformatics data

[0303] The cloned rat 648 cDNA contains an ORF coding for a putative protein that is 88% identical to the published human PLOD2 sequences. The least conserved sequences are within the signal peptide, however its functional features are completely preserved. The cloned human cDNA is almost identical to published human PLOD2 sequence. The word "almost" in the previous sentence stems from the fact that both in human and in rat cDNA species cloned in the inventors' laboratory a stretch of amino acids between positions 501-521 of published sequence PLOD2 sequence was absent. Therefore, the present PLOD2 variants are differentially spliced. Both rat and human homologues were amplified from RNA extracted from glioma cell lines cultured in hypoxic conditions.

Literature Review

[0304] Lysyl hydroxylases are the enzymes that catalyze the formation of hydroxylysine in collagens and other proteins with collagen-like amino-acid sequences, by the hydroxylation of lysine residue in X-K-G sequences. The hydroxylysine residues have two important functions: (1) serve as sites of

attachment of carbohydrate units, and (2) they are essential for the stability of the intermolecular collagen crosslinks. Congenital deficiency of lysyl hydroxylase in humans leads to increased solubility of collagens and, consequently, to numerous defects in organization of connective tissue in various organs. There are three known isoforms of lysyl hydroxylase, encoded by different genes. In humans, PLOD2 was found to be highly expressed in pancreas, skeletal muscle, heart and placenta (by Northern blot). Nothing is known either about the regulation of PLOD2 expression by hypoxia or about its involvement in angiogenesis and tumorigenesis. Induction of PLOD2 by hypoxia can probably account for hypoxia-induced tissue fibrosis. Indeed, specific lysyl hydroxylase inhibitor, minoxidil, was able to suppress both cellular collagen production and fibroblasts proliferation (Murad et al, 1987; Saika et al, 1995). There were suggestions in literature to use modified lysyl hydroxylase inhibitor for treatment of vitreoretinopathy (Handa et al, 1993).

<u>Analysis of Alternatively Spliced Versions of Gene 648</u>

[0305] In order to establish whether the observed alternative splicing of PLOD2 is regulated by hypoxia, a set of PCR primers were synthesized that flank the alternatively spliced region. The expected sizes of RT-PCR products are:

216 bp, for published sequence and 156 bp, for the present sequence. Semi-quantitative RT-PCR was performed on RNA template extracted from human glioma A172 cell culture in either normoxia or in hypoxia for 4 and 16 hours. The obtained results clearly demonstrate that both PLOD2 forms are hypoxia regulated, but the form of the invention appears only in hypoxic conditions.

Testing Potential Pro- and Antiapoptotic Activity in Transient Transfection Assays

[0306] pcDNA3-648 was transiently co-transfected together with pcDNA3-GFP in Hela and 293 cells. 24 and 48 hours later the cells were fixed and stained with DAPI. No apoptotic effect was observed in the transfected cells. In order to evaluate the anti-apoptotic properties of the 648 protein, a co-transfection assay was conducted using the pcDNA3-GFP and the FAS plasmids. No anti-apoptotic effect was observed.

Obtaining Stable Cell Clones Overexpressing 648 cDNA

[0307] C6 were stably transfected with 5 µg of the pcDNA3-648 plasmid. Following G418 selection the level of expression was measured using Northern blot analysis in comparison to its level in C6 cells after 16 hours under hypoxic conditions. Out of 18 independent clones from the pcDNA3-648 transfection, no one was positive.

In situ Hybridization Analysis

Retinopathy Model

[0308] Probe 648 demonstrates clear hybridization signal throughout the inner nuclear layer of "hypoxic" pup's retina while "normoxic" retina is negative for the expression. No hybridization signal was detected in adult retina.

[0309] In mouse embryo sections hybridization signal was detected in some apoptotic cells in the roof of the fourth brain ventricle and in developing retina ganglia, where expressing cells had no apoptotic features.

[0310] Multi-tissue block hybridization shows expression of 648 gene (rat PLOD2) in visceral smooth muscles in oviduct, uterus, stomach and intestine. Vascular smooth muscles do not display hybridization signal.

[0311] The most prominent cell type hybridizing to 648 probe in the ovary are granulosa cells of larger secondary follicles. No hybridization signal is detected in granulosa cells of primary and small secondary follicles. Significantly, hybridization signal is weakened in postovulatory follicles and completely disappears in corpora lutea. This shows that expression in granulosa cells is established at later stages of follicular maturation and it is abruptly down-regulated upon ovulation and the onset of conversion into lutein cells. On the other hand, follicular

involution is not accompanied by the changes in 648 expression since strong hybridization signal is preserved in granulosa cells of atretic follicles.

[0312] Weak hybridization signal can be seen in some stromal cells surrounding large secondary follicles and corpora lutea as well as in cells of theca internal of secondary follicles. Prominent signal is found in "interstitial glands". This shows distinct regulation of 648 expression in theca cells undergoing "luteinization" in different locations: it is down regulated in corpora lutea but preserved or even up-regulated in interstitial glands.

[0313] As to the germinal cells, an oocyte that expresses 648 was found only in one primary follicle while many other primary and secondary follicles had no hybridization signal. This shows a transient expression of 648 in oocytes at some stage of their development.

[0314] Discrepancy in the hybridization patterns of human (published) and rat PLOD2 (648) genes is explained by different sensitivities of different detection methods (Northern blot vs. *in situ* hybridization). The rat probe used in the present invention does not span an alternatively spliced region.

EXAMPLE 6: 24D4 (SEQ ID NO:14)

Northern Blot Analysis

[0315] Expression of gene 24D4 is down-regulated after 16 hours of hypoxia. On Northern blots, it appears as a single 1.5 Kb mRNA species.

Cloning

[0316] A partial 24D4 human cDNA clone was isolated from A172 cDNA library. The available sequence is 1486 bp long and contains an N-terminal truncated ORF (bp 1-396), encoding the peptide of SEQ ID NO:15.

Bioinformatic Analysis

[0317] The sequence has no analogs in public databases. The available protein sequence contains three consequent Zn-finger motifs, all of C2H2 type (aa 52-72, 80-100 and 108-128). Zinc finger domains of this type are usually found in nucleic acid-binding proteins.

EXAMPLE 7: 77H4 (SEQ ID NO:16)

Northern Blot Analysis

[0318] Expression of gene 77H4 is up-regulated after 16 hours of hypoxia. On Northern blots, it appears as a single mRNA species 0.6-0.7 Kb in size.

Cloning

[0319] Several EST cDNA clones from public databases, corresponding to clone 77H4, were sequenced. All clones

possess a poly A tail and a polyadenylation signal at their 3' end.

Bioinformatic Analysis

[0320] Gene 77H4 (SEQ ID NO:16) encodes a 360 bp protein (SEQ ID NO:17). An exhaustive search was performed of public databases for all 77H4-related sequences. Several independent contigs were identified in TIGR THC database. All of them are not completely identical to one another and contain nucleotide deletions of various length. This shows a certain variability in 77H4 nucleotide sequence.

[0321] Recently, a novel steroid receptor transcriptional coactivator, SRA, was found to be present as an RNA molecule in the transcription activating complex SRC-1 (Lanz et al, 1999). Although no similarity was found between clone 77H4 and SRA RNA on the sequence level, several characteristic features seem to be shared by both sequences:

- both mRNAs, 77H4 and SRA, are approximately of the same size - 0.7 Kb;
- sequencing multiple cDNA clones corresponding to either mRNA revealed extensive variability in certain regions;
- hybridization signals of both mRNA, therefore, appear as fuzzy bands on Northern blots;
- neither mRNA exhibit characteristics of protein.

[0322] Therefore, the 77H4 cDNA clone has similar to SRA function and can serve a coactivator in some transcriptional complexes.

EXAMPLE 8: 14G2 (SEQ ID NO:18)

Northern Blot Analysis

[0323] Expression of gene 14G2 is regulated within 16 hours of hypoxia. On Northern blots, it appears as a single mRNA species.

Cloning

[0324] A partial 14G2 human cDNA clone was isolated. The available sequence was then characterized and cloned as shown in SEQ ID NO:18.

EXAMPLE 9: 29F3 (SEQ ID NO:19)

Northern Blot Analysis

[0325] Expression of gene 29F3 is regulated within 16 hours of hypoxia. On Northern blots, it appears as a single mRNA species.

Cloning

[0326] A partial 29F3 human cDNA clone was isolated. The available sequence was then characterized and cloned as shown in SEQ ID NO:19.

[0327] Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. Full citations for the

publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

[0328] The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

[0329] Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the described invention, the invention can be practiced otherwise than as specifically described.

[0330] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose

of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever structural, physical, chemical or electrical element or structure, or whatever method step, which may now or in the future exist which carries out the recited function, whether or not precisely equivalent to the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same functions can be used; and it is intended that such expressions be given their broadest interpretation.

[0331] The sequence list attached hereto is hereby incorporated by reference.

REFERENCES

Abe et al, "Specific inhibition of influenza virus RNA polymerase and nucleoprotein gene expression by liposomally encapsulated antisense phosphorothioate oligonucleotides in MDCK cells", Antivir Chem Chemother 9:253-262 (1998)

Adams et al, "Complementary DNA sequencing: expressed sequence tags and human genome project", Science 252(5013):1651-1656 (1991)

Agrawal S, "Antisense oligonucleotides: towards clinical trials", Trends Biotechnol 14(10):376-387 (1996)

An et al, "Stabillization of wild-type p53 by Hypoxia-Inducible Factor-1α", Nature 392:405-408 (1998)

Anazodo et al, "Relative levels of inhibition of p24 gene expression by different 20-mer antisense oligonucleotide sequences targeting nucleotides + 1129 to + 1268 of the HIV-1 gag genome: an analysis of mechanism", Biochem Biophys Res Commun 229(1):305-309 (1996)

Ausubel et al, Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1989)

Ausubel et al (Eds), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (New York, 1994-2000)

Bai et al, "Gene transfer to vein graft wall by HVJ-liposome method: time course and localization of gene expression", Ann Thorac Surg 66:814-819 (1998)

Barone et al, "CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest", Genes Dev 8(4):453-464 (1994)

Batchvarova et al, "Inhibition of adipogenesis by the stress induced protein CHOP (gadd 153)", EMBO J 14:4654-4661 (1995)

Baudet et al, "1,25-Dihydroxyvitamin D3 induces programmed cell death in a rat glioma cell line", J Neurosci Res 46(5):540-550 (1996)

Belecky-Adams et al, "Intragenic sequences are required for cell type-specific and injury-induced expression of the rat peripherin gene", J Neurosicence 13:5056-5065 (1993)

Blagosklonny et al, "p53 inhibits Hypoxia-Inducible Factor-stimulated transcription", J Biol Chem 273:11995-11998 (1998)

Bochot et al, "Liposomes dispersed within a thermosensitive gel: a new dosage form for ocular delivery of oligonucleotides", Pharm Res 15:1364-1369 (1998)

Borrebaeck, Antibody Engineering - A Practical Guide, W.H. Freeman and Co., 1992

Bouck et al, "How tumors become angiogenic", Adv Cancer Res 69:135-174 1(996).

Boutin et al, "Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells," Molec Endocrinol 3(9):1455-1461 (1989)

Braren et al, "Use of the EST database resource to identify and clone novel mono(ADP-ribosyl)transferase gene family members", Adv Exp Med Bio 419:163-168 (1997).

Braun et al, "A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts", EMBO J 8(3):701-709 (1989)

Bunn et al, "Oxygen sensing and molecular adaptation in hypoxia",Physiol Rev 76:839-885 (1996).

Burke et al, "Preparation of Clone Libraries in Yeast Artificial-Chromosome Vectors" in Methods in Enzymology, Vol. 194, Guide to Yeast Genetics and Molecular Biology, eds. Guthrie et al, Academic Press, Inc., Chap. 17, pp. 251-270 (1991).

Cao et al, "Lymphotactin gene-modified bone marrow dendritic cells act as more potent adjuvants for peptide delivery to induce specific antitumor immunity", J Immunol 161:6238-6244 (1998)

Calabretta et al, "Antisense strategies in the treatment of leukemias", Semin Oncol 23:78 (1996)

Cantor et al, " Ribozyme cleaves rex/tax mRNA and inhibits bovine leukemia virus expression", Proc Natl Acad Sci, 90(23):10932 (1993)

Capaccioli et al, "Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum", Biochem Biophys Res Comm 197:818-825 (1993)

Capecchi MR, "Altering the genome by homologous recombination" Science 244:1288-1292 (1989).

Carmeliet et al, "Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumor angiogenesis", Nature 394(6692):485-490 (1998)

Chan et al,"Molecular cloning and localization to chromosome 6 of mouse INT1L1 gene", Somatic Cell Molec Genet 15(6):555-562 (1989)

Chen et al, "Inhibition of HIV-1 replication by novel multitarget ribozymes", Ann NY Acad Sci 660:271-273 (1992)

Chen et al, "Down-regulation of gadd153 by c-myc in rat fibroblasts and its effect on cell growth and radiation-induced apoptosis", Oncogene 13(8):1659-1665 (1996)

Clark et al, "Apoptosis-suppressor gene bcl-2 expression after traumatic brain injury in rats", J Neurosci 17: 9172-9182 (1997)

Crooke ST, "Progress in antisense therapeutics", Hematol Pathol 2:59 (1995)

Croyle et al, "Development of a highly efficient purification process for recombinant adenoviral vectors for oral gene delivery", Pharm Dev Technol 3(3)365-372 (1998)

Daniel et al, "Retroviral transfer of antisense sequences results in reduction of C-Abl and induction of apoptosis in hemopoietic cells", J Biomed Sci 5:383-394 (1998)

Davies et al, "Targeted alterations in yeast artificial chromosomes for inter-species gene transfer", Nucleic Acids Research, Vol. 20, NO 11, pp. 2693-2698 (1992).

Davis et al (eds.), Basic Methods in Molecular Biology, Elsevier Press, NY (1986)

Dickinson et al, "High frequency gene targeting using insertional vectors", Human Molecular Genetics, 2(8)1299-1302 (1993)

Dor et al, "Ischemia-driven angiogenesis", Trends Cardiovasc Med 7:289-294 (1997)

Duff et al, "Insertion of a pathogenic mutation into a yeast artificial chromosome containing the human APP gene and expression in ES cells", Research Advances in Alzheimer's Disease and Related Disorders, 1995.

Duke et al, "Cell Suicide in Health and Disease", Sci Am 275(6):80-87 (1996)

Eckstein F, "Nucleoside Phosphorothioates", Ann Rev Biochem 54:367-402 (1985)

Edinger et al, "Use of GPR1, GPR15, and STRL33 as coreceptors by diverse human immunodeficiency virus type 1 and simian immunodeficiency virus envelope proteins", Virology 249:367-378 (1998)

Faerman et al, "Transgenic mice: production and analysis of expression", Methods Cell Biol 52:373-403 (1997)

Fang et al, "A packaging system for SV40 vectors without viral coding sequences", Anal Biochem 254:139-143 (1997)

Felgner PL, "Nonviral Strategeies for Gene Therapy", Sci Am, 276(6):102-106 (1997)

Flanagan WM, "Antisense comes of age", Cancer Metastasis Rev, 17(2):169-176 (1998)

Fleming et al, "The effects of nutrient deprivation and differentiation on the expression of growth arrest genes (gas and gadd) in F9 embryonal carcinoma cells", (1998) 330:573-579 (1998)

Forance et al, "Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents", Mol Cell Biol 9:4196-4203 (1989)

Foster et al, "Identification of a novel human Rho protein with unusual properties: GTPase deficiency and in vivo farnesylation", Mol Cell Biol 16(6):2689-2699 (1996)

Galizzi et al, Molecular cloning of a cDNA encoding the human interleukin 4 receptor", Int Immunol 2(7):669-675 (1990)

Gennaro AR (ed.), Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA, 1990, pp 1521-1712

Gerster et al, "Quantitative analysis of modified antisense oligonucleotides in biological fluids using cationic nanoparticles for solid-phase extraction", Anal Biochem 262:177-184 (1998)

Gewirtz "Oligodeoxynucleotide-based therapeutics for human leukemias", Stem Cells Dayt 11:96 (1993)

Gordon et al, 1989

Gould et al, "Use of the DNA polymerase chain reaction for homology probing: isolation of partial cDNA or genomic clones encoding the iron-sulfur protein of succinate dehydrogenase from several species", Proc Nat Acad Sci USA 86(6):1934-1938 (1989)

Graeber et al, "Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours", Nature 379(6560):88-91 (1996)

Griscelli et al, "Heart-specific targeting of beta-galactosidase by the ventricle-specific cardiac myosin light chain 2 promoter using adenovirus vectors", Hum Gene Ther, 9:1919-1928 (1998)

Guang-Lin et al, "Adenovirus-mediated gene transfer of CTLA4IG gene results in prolonged survival of heart allograft", Transplant Proc, 30:2923-2924 (1998)

Guinot et al, "Antisense oligonucleotides: a new therapeutic approach", Pathol Biol, 46:347-354 (1998)

Gutkowska et al, "Circulating forms and radioimmunoassay of atrial natriuretic factor", Endocrinol Metab Clin North Am 16(1):183-198 (1987)

Hampel et al, "RNA catalytic properties of the minimum (-)sTRSV sequence", Biochemistry 28(12):4929-33 (1989)

Hampton et al, "Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis", FEBS Lett 14: 552-556 (1997)

Hanania et al, "Recent advances in the application of gene therapy to human disease", Am J Med 99:537 (1995)

Hanahan et al, "Patterns and Emerging Mechanisms of Angiogenic Switch During Tumorigenesis"; Cell 86:353-364 (1996)

Handa et al, "Minoxidil inhibits ocular cell proliferation and lysyl hydroxylase activity", Invest Ophthalmol Vis Sci 34(3)567-575 (1993)

Harlow et al, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1988)

He et al, "Molecular cloning of androgen receptors from divergent species with a polymerase chain reaction technique: complete cDNA sequence of the mouse androgen receptor and isolation of androgen receptor cDNA probes from dog, guinea pig and clawed frog", Biochem Biophys Res Comm 171(2):697-704 (1990)

Hemmi et al, "The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures", Hum Gene Ther 9:2363-2373 (1998)

Herskowitz I, "Functional inactivation of genes by dominant negative mutations", Nature 329(6136):219-222 (1987)

Higuti et al, "Molecular cloning of cDNA for the import precursor of human subunit B of H(+)-ATP synthase in mitochondria", Biochem Biophys Res Comm 178(3):1014-1020 (1991)

Hockenbery et al, "Bcl-2 functions in an antioxidant pathway to prevent apoptosis", Cell 75:241-251 (1993)

Hofmann et al, "Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette", Proc Natl Acad Sci 93(11):5185-5190 (1996)

Hollander et al, "Analysis of the mammalian gadd45 gene and its response to DNA damage", J Biol Chem 268:24385-24393 (1993)

Hsieh et al, "Chromosome localization and cDNA sequence of murine and human genes for ras p21 GTPase activating protein (GAP)," Somat Cell Mol Genet 15(6):579-590 (1989)

Huston et al, "Protein engineering of single-chain Fv analogs and fusion proteins" in Methods in Enzymology (JJ Langone, ed.; Academic Press, New York, NY) 203:46-88 (1991)

Huxley et al, "The human HPRT gene on a yeast artificial chromosome is functional when transferred to mouse cells by cell fusion", Genomics 9:742-750 (1991)

Imamura et al, "Molecular cloning and primary structure of rat thyroxine-binding globulin", Biochemistry 30(22):5406-5411 (1991)

Itakura et al, "Both HPV and carcinogen contribute to the development of resistance to apoptosis during oral carcinogenesis", Int J Oncol 16(3):591-597 (2000)

Iwata et al, "Structure of the mouse tyrosine hydroxylase gene", Biochem Biophys Res Comm 182(1):348-354 (1992)

Iyer et al, J Org Chem 55:4693-4699 (1990)

Jakobovits et al, "Germ-line transmission and expression of a human-derived yeast artificial chromosome", Nature, Vol. 362, pp. 255-261 (1993)

Jakubiczka et al, "A bovine homologue of the human TSPY gene", Genomics 17(3):732-735 (1993)

James-Pederson et al, "Flanking and intragenic sequences regulating the expression of the rabbit alpha-globin gene", J Bio. Chem 270:3965-3973 (1995)

Jeung et al, "Molecular cloning of the full-length cDNA encoding the human calbindin-D9k", FEBS Lett 307(2):224-228 (1992)

Johnson et al, "Construction of single-chain Fvb derivatives of monoclonal antibodies and their production in *Escherichia coli* in Methods in Enzymology (JJ Langone, ed.; Academic Press, New York, NY) 203:88-99 1991

Johnstone et al, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982

Jogi et al, "Patched 2, located in 1p32-34, is not mutated in high stage neuroblastoma tumors", Int J Oncol 16:943-949 (2000)

Joseph et al, "Optimization of an anti-HIV hairpin ribozyme by *in vitro* selection", J Biol Chem 268:24515 (1993)

Kähäri et al, "Deletion analyses of 5'-flanking region of the human elastin gene. Delineation of functional promoter and regulatory cis-elements", J Biol Chem 265(16):9485-9490 (1990)

Kanamaru et al, "Biological effects and cellular uptake of c-myc antisense oligonucleotides and their cationic liposome complexes", J Drug Target 5:235-246 (1998)

Kim et al, "A new non-viral DNA delivery vector: the terplex system", J Controlled Release 53(1-3):175-82 (1998)

Kim et al, "Introduction of gadd153 gene into gastric cancer cells can modulate sensitivity to anticancer agents in association with apoptosis", Anticancer Res 19:1779-1783 (1999)

Kita et al, "Growth inhibition of human pancreatic cancer cell lines by anti-sense oligonucleotides specific to mutated K-ras genes", Int J Cancer 80:553-558 (1999)

Kondo et al, "Modulation of apoptosis by endogenous Bcl-xL expression in MKN-45 human gastric cancer cells", Oncogene 17:2585-2591 (1998)

Korabiowska et al, "Loss of growth arrest DNA damage genes expression in oral melanomas", In Vivo 13:483-485 (1999)

Kumar et al, "Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes", Microbiol Mol Biol Rev, 62:1415-1434 (1998)

Lamb et al, "Introduction and expression of the 400 kilobase *precursor amyloid protein* gene in transgenic mice", Nature Genetics, Vol. 5, pp. 22-29 (1993).

Lane DP, "Cancer. p53, guardian of the genome", Nature 358(6381):15-6 (1992)

Langner et al, "Viral particles with heterologous binding motifs. An approach to specifically alter the tropism of the B-lymphotropic papovavirus", Adv Exp Med Biol 451:415-422 (1998)

Lanz et al, "A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex" Cell 97:1 17-27 (1999)

Lavitrano et al, "Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice", Cell 57(5):717-723 (1989)

Lawrence et al, "Overexpression of Bcl-2 with herpes simplex virus vectors protects CNS neurons against neurological insults in vitro and in vivo", J Neurosci 16:486-496 (1996)

Lefebvre-d'Hellencourt et al, "Immunomodulation by cytokine antisense oligonucleotides", Eur Cytokine Netw 6:7 (1995)

Lev-Lehman et al, "Antisense Oligomers *in vitro* and *in vivo*" in Antisense Therapeutics, Cohen et al, eds (Plenum Press, New York) (1997)

Libert et al, "Cloning and functional characterization of a human A1 adenosine receptor", Biochem Biophys Res Comm 187(2):919-926 (1992)

Lo, 1983

Mansour SL, "Gene targeting in murine embryonic stem cells: introduction of specific alterations into the mammalian genome", Genet Anal Tech Appl 7(8):219-227 (1990)

Martinou et al, "Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia", Neuron 13:1017-1030 (1994)

Meinkoth et al, "Hybridization of nucleic acids immobilized on solid supports", Anal Biochem 138:267-284 (1984)

Meiri et al, "Memory and long-term potentiation (LTP) dissociated: normal spatial memory despite CA1 LTP elimination with Kv1.4 antisense", Proc Natl Acad Sci, 95:15037-15042 (1998)

Mernaugh et al, "An overview of phage-displayed recombinant antibodies" in Molecular Methods In Plant Pathology (Singh et al, eds.), CRC Press Inc., Boca Raton, FL (1995) pp. 359-365

Meyer et al, "Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides," J Biol Chem 273(25):15621-15627 (1998)

Mishell et al (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980)

Mizuguchi et al, "Efficient gene transfer into mammalian cells using fusogenic liposome", Biochem Biophys Res Commun 218:402-407 (1996)

Murad et al, "Suppression of fibroblast proliferation and lysyl hydroxylase activity by minoxidil", J Biol Chem 262(25):11973-11978 (1987)

Muranishi et al, "Lipophilic peptides: synthesis of lauroyl thyrotropin-releasing hormone and its biological activity", Pharm Research 8:649-652 (1991)

Nahmias et al, "Molecular characterization of the mouse beta 3-adrenergic receptor: relationship with the atypical receptor of adipocytes", EMBO J 10(12):3721-3727 (1991)

Nakamura et al, "A comparison of in vivo gene delivery methods for antisense therapy in ligament healing", Gene Ther 5:1455-1461 (1998)

Narumi et al, "Adenovirus vector-mediated perforin expression driven by a glucocorticoid-inducible promoter inhibits tumor growth in vivo", Am J Respir Cell Mol Biol 19:936-941 (1998)

Nicholson et al, "Caspases: killer proteases", Trends Biochem Sci 22:299-306 (1997)

Nishida et al, "Adenovirus-mediated gene transfer to nucleus pulposus cells. Implications for the treatment of intervertebral disc degeneration", Spine 23:2437-2442, 1998

Noguchi et al, "Membrane fusion plays an important role in gene transfection mediated by cationic liposomes", FEBS Lett 433:169-173 (1998)

Obeso et al, "A Hemangioendothelioma-Derived Cell Line: Its Use as a Model for the Study of Endothelial Cell Biology", Laboratory Investigation 83:259-264 (1990)

Okubo et al, "Complementary DNA sequence (EST) collections and the expression information of the human genome", FEBS Lett 403:225-229 (1997)

Oro et al, "The Drosophila gene knirps-related is a member of the steroid-receptor gene superfamily", Nature 336(6198):493-496 (1988)

Paglia et al, "Gene transfer in dendritic cells, induced by oral DNA vaccination with Salmonella typhimurium, results in protective immunity against a murine fibrosarcoma", Blood 92(9):3172-3176 (1998)

Pearson et al, "Expression of the human β-amyloid precursor protein gene from a heast artificial chromosome in transgenic mice", Proc Natl Scad Sci USA 90:10578-10582 (1993)

Pederson et al, "Combined cytosine deaminase expression, 5-fluorocytosine exposure, and radiotherapy increases cytotoxicity to cholangiocarcinoma cells", J Gastrointest Surg 2:283-291 (1998)

Peng Ho et al, "Modification of phosphorothioate oligonucleotides yields potent analogs with minimal toxicity for antisense experiments in the CNS", Brain Res Mol Brain Res 62(1):1-11 (1998)

Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons, New York (1988)

Phelan et al, "The human mammary-derived growth inhibitor (MDGI) gene: genomic structure and mutation analysis in human breast tumors", Genomics 34:63-68 (1996)

Potier et al, "The human glutamate receptor cDNA GluR1: cloning, sequencing, expression and localization to chromosome 5", DNA Seq 2(4):211-218 (1992)

Price et al, 1992

Quattrone et al, "Enhancing antisense oligonucleotide intracellular levels by means of cationic lipids as vectors", Biochemica 1:25-29 (1995)

Reaume et al, " Enhanced amyloidogenic processing of the beta-amyloid precursor protein in gene-targeted mice bearing the Swedish familial Alzheimer's disease mutations and a "humanized" Abeta sequence", J Biol Chem 271(38):23380-8 (1996)

Reecy et al, "Multiple regions of the porcine alpha-skeletal actin gene modulate muscle-specific expression in cell culture and directly injected skeletal muscle", Anim Biotechnol 9:101-120 (1998)

Reed JC, "Bcl-2 and the regulation of programmed cell death", J Cell Biol 124:1-6 (1994)

Rothstein, "Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast" in Methods in Enzymology, Vol. 194, "Guide to Yeast Genetics and Molecular Biology", eds. C. Guthrie and G. Fink, Academic Press, Inc., Chap. 19, pp. 281-301 (1991).

Saika et al, "Effect of lysyl hydroxylase inhibitor, minoxidil, on ultrastructure and behavior of cultured rabbit subconjunctival fibroblasts", Graefes Arch Clin Exp Ophthalmol 233(6):347-353 (1995)

Sambrook et al (Eds), Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, (NY, 1989, 1992)

Sarver et al, Gene Regulation and Aids, pp. 305-325 (1990)

Scanlon et al, "Oligonucleotides-mediated modulation of mammalian gene expression", FASEB J 9:1288 (1995)

Schedl et al, "A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice", Nature, Vol. 362, pp. 258-261 (1993).

Schena et al, "Parallel Human Genome Analysis: Microarray-based Expression Monitoring of 1000 genes", Proc Natl Acad Sci USA 93(20):10614-10619 (1996)

Schmidt-Kastner et al "Pixel-based image analysis of HSP70, GADD45 and MAP2 mRNA expression after focal cerebral ischemia: hemodynamic and histological correlates" Brain Res Mol Brain Res 63:79-97 (1998)

Schwarzenberger et al, "IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for *in vivo* evaluation of cytokines", J Immunol 161:6383-6389 (1998)

Shaw et al, "Modified deoxyoligonucleotides stable to exonuclease degradation in serum", Nucleic Acids Res 19:747-750 (1991)

Shibata et al, "Caspases determine the vulnerability of oligodendroctes in the ischemic brain", J Clin. Invest 106:643-653 (2000)

Shimayama et al, "Cleavage of the highly conserved hairpin-loop region of HIV-1 by synthetic ribozymes", Nucleic Acids Symp Ser 29:177-178 (1993)

Shoji et al, "Enhancement of anti-herpetic activity of antisense phosphorothioate oligonucleotides 5' end modified with geraniol", J Drug Target 5:261-273 (1998)

Shore et al, "Ribozyme-mediated cleavage of the BCRABL oncogene transcript: in vitro cleavage of RNA and in vivo loss of P210 protein-kinase activity", Oncogene 8:3183-3188 (1993)

Smith et al, "Induction of the p53 regulated protein gadd45 with proliferating cell nuclear antigen", Science 266:1376-1380 (1994)

Soni et al, "Biodistribution, stability, and antiviral efficacy of liposome-entrapped phosphorothioate antisense oligodeoxynucleotides in ducks for the treatment of chronic duck hepatitis B virus infection", Hepatology 28:1402-1410 (1998)

Soukchareun et al, "Use of Nalpha-Fmoc-cysteine(S-thiobutyl) derivatized oligodeoxynucleotides for the preparation of oligodeoxynucleotide-peptide hybrid molecules", Bioconjug. Chem 9(4):466-475 (1998)

Spitzer et al, "Inhibition of deoxynucleases by phosphorothioate groups in oligodeoxyribonucleotides", Nucleic Acids Res 18:11691-11704 (1988)

Stites et al (eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994)

Stix, G, "Shutting down a gene. Antisense drug wins approval", Sci Am 279(5):46, 50 (1998)

Strauss et al, "Germ line transmission of a yeast artificial chromosome spanning the murine α_1 (I) collagen locus", Science 259:1904-1907 (1993)

Stridh et al, "Cytochrome c release and caspase activation in hydrogen peroxide-and tributyltin-induced apoptosis", FEBS Lett 429:351-355 (1998)

Sugano et al, "Cyclosporin A inhibits H_2O_2- induced apoptosis of human fibroblasts",FEBS Lett 447:274-276 (1999)

Sugawa et al, "An antisense EGFR oligodeoxynucleotide enveloped in Lipofectin induces growth inhibition in human malignant gliomas *in vitro*", J Neurooncol 39:237-244 (1998)

Sullivan, 1994

Sumimoto et al, " Complementary DNA for the mouse homolog of the small subunit of human cytochrome b558", Biochem Biophys Res Comm 165(2):902-906 (1989)

Symons RH, "Self-cleavage of RNA in the replication of small pathogens of plants and animals", Trends Biochem Sci 14(11):445-450 (1989)

Symons RH, "Small catalytic RNAs", Annu Rev Biochem 61:641-671 (1992)

Takekawa et al, "A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK", Cell 95:521-530 (1998)

Teoh et al, "Adenovirus vector-based purging of multiple myeloma cells", Blood, 92:4591-4601 (1998)

Testoni et al, "A new method of "in-cell reverse transcriptase-polymerase chain reaction" for the detection of BCR/ABL transcript in chronic myeloid leukemia patients", Blood 87(9):3822-3827 (1996)

Thompson et al, 1989

Uhlenbeck OC, "A small catalytic oligoribonucleotide", Nature 328(6131):596-600 (1987)

Uhlmann et al, "Antisense Oligonucleotides: A New Therapeutic Principle", Chem Rev 90(4):543-584 , (1990)

van der Putten et al, "Efficient insertion of genes into the mouse germ line via retroviral vectors", Proc Natl Acad Sci USA 82(18):6148-6152 (1985)

Velasco-Miguel et al, "PA26, a novel target of p53 tumor suppressor and member of the GADD family of DNA damage and growth arrest inducible genes", Oncogene 18(1):127-137 (1999)

Waelti et al., "Delivery to cancer cells of antisense L-myc oligonucleotides incorporated in fusogenic, cationic-lipid-reconstituted influenza-virus envelopes (cationic virosomes)", Int J Cancer 77:728-733 (1998)

Wagner, RW "Gene inhibition using antisense oligodeoxynucleotides", Nature 372(6504):333-335 (1994)

Wang et al, "3-Hydroxy-3-methylglutaryl coenzyme A lyase (HL): cloning and characterization of a mouse liver HL cDNA and subchromosomal mapping of the human and mouse HL gene", Mamm Genome 4(7):382-387 (1993)

Wang, "Cyclic peptides incorporating 4-carboxyphenylalanine and phosphotyrosine are potent inhibitors of pp60 (c-)(src)", J Controlled Release 53:39-48 (1998)

Wang et al, "Monitoring gene expression profile changes in ovarian carcinomas using cDNA microarray", Gene 229(1-2):101-108 (1999)

Watson et al, Recombinant DNA, Scientific American Books, New York

Weissig et al, "DQAsomes: a novel potential drug and gene delivery system made from Dequalinium", Pharm Res 15:334-337 (1998)

Wenger et al, "Oxygen(es) and the hypoxia-inducible factor-1", Biol Chem 378(7):609-616 (1997)

Woolf et al, "The stability, toxicity and effectiveness of unmodified and phosphorothioate antisense oligodeoxynucleotides in *Xenopus* oocytes and embryos", Nucleic Acids Res 18:1763-1769 (1990)

Yakubov et al, "Mechanism of oligonucleotide uptake by cells: involvement of specific receptors?", Proc Natl Acad Sci USA 86(17):6454-6458 (1989)

Yang et al, 1998

Yeh et al, "Inhibition of BMP receptor synthesis by antisense oligonucleotides attenuates OP-1 action in primary cultures of fetal rat calvaria cells", J Bone Miner Res 13:1870-1879 (1998)

Yotsuyanagi et al, "Cationic liposomes in gene delivery", Nippon Rinsho 56:705-712 (1998)

Zacharia et al, "New reduced peptide bond substance P agonists and antagonists: effects on smooth muscle contraction", Eur J Pharmacol 203:353-357 (1991)

Zhan et al, "Abrogation of p53 function affects gadd gene response to DNA base-damaging agents and starvation" DNA Cell Biol 15:805-815 (1996)

Zhao et al, "Generating loss-of-function phenotypes of the fushi tarazu gene with a targeted ribozyme in Drosophila", Nature 365:448-451 (1993)

Zweiger et al, "From Expressed Sequence Tags to "epigenomics": An Understanding of Disease Processes", Curr Opin Biotechnol 8(6):684-687 (1997)

AC004283: Drosophila melanogaster; DNA sequence (P1 DS05557 (D152)), complete sequence; LOCUS: AC004283; submitted 29 August 1998

AF131826: Homo sapiens clone 24945; mRNA sequence, partial cds; LOCUS: AF131826; submitted 12 March 1999

AAB37671 (1703624): LOCUS: AAB37671; submitted 04 December 1996

P52199: Rho-Related GTP-Binding Protein RHOE (RHO8); LOCUS RHOE-HUMAN; submitted 15 December 1998

S73591: MG068 homolog D02_orf346-Mycoplasma pneumonia (strain ATCC 29342); LOCUS: S73591; originally submitted 27 February 1997; last revision 07 December 1998